Early life experience

neuroendocrine adaptations to maternal absence

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The statistician who supposes that his main contribution to the planning of an experiment will involve statistical theory, finds repeatedly that he makes his most valuable contribution simply by persuading the investigator to explain why he wishes to do the experiment, by persuading him to justify the experimental treatments, and to explain why it is that the experiment, when completed, will assist him in his research.

Gertrude M. Cox.

Voor Petra,
mijn allerliefste

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Preface

Preface

An adverse early life event is considered a risk factor for stress-related psychiatric disorders in genetically predisposed individuals, probably because of its lasting effect on susceptibility to stress. The objective of this thesis research was to examine in the mouse CD1 strain the immediate and permanent effects of an adverse early experience on the neuroendocrine stress system. For this purpose the hypothalamic-pituitary-adrenal (HPA) axis was examined of mouse pups that were refrained from maternal care, a laboratory model for neglect mimicking aspects of abuse. The data show that the infants' stress response system readily adapts to daily repeated 8 hours of maternal separation, but that it continues to respond to a novelty stressor. The rapid adaptation to repeated maternal absence seems rather due to the ability to predict return of the mother than to adjust metabolism to episodic food deprivation. If maternal separation was extended to a single episode of 24 hours the immediate outcome was more profound but transient, although subtle effects on stress reactions and cognitive performance did persist. The findings demonstrate the amazing plasticity of the newborn brain and provide a basis to study the mechanistic underpinning of vulnerability or resilience to psychopathology.

Chapter 1

Introduction

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1.1 Stress

The term stress, as used in this thesis, was originally introduced by Hans Selye as "the biological phenomenon of a disrupted homeostasis" [174]. Since the 1950s this field of research has evolved substantially and McEwen recently presented this definition of stress as follows [125]: "Stress may be defined as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioural responses. In biomedical terms, stress often refers to situations in which adrenal glucocorticoids and catecholamines are elevated because of an experience".

In this chapter I will describe the role of the hypothalamic-pituitary-adrenal axis (HPA axis) in response to stress and focus on factors controlling this axis' responsivity in early life. In humans, adverse events occurring early in life can permanently alter the set point of HPA axis activity and affect the onset and termination of the HPA axis response to stress with consequences for resilience and coping. To draw a parallel to the human situation animal models have been developed to study this so-called programming of the HPA axis. I will first describe the normal HPA axis response to stress (***section 1.1***) and will then focus on the effects of adverse early life experiences in humans (***section 1.2***). Finally, this chapter will give an overview of normal rodent HPA axis development (***section 1.3***), the consequences of disrupting the axis in rodents (***section 1.4.***) and present a scope and outline of this thesis (***section 1.5***).

1.1.1 The HPA axis response to stress

When an organism experiences a challenging situation threatening to disrupt homeostasis, which is stress, the HPA axis is activated (see ***Figure 1.1***). The central response to stress is a highly integrated process in which diverse neuronal systems are involved [38, 77]. Both physical and psychological stressors can activate the central component of the HPA axis, although through different neuronal pathways. Physical stressors, such as for example infections, temperature changes and dehydration, mainly activate catecholaminergic systems located in the brainstem area, like the locus coeruleus and the nucleus tractus solitarii. Psychological stressors, on the other hand, mainly depend on activation of limbic structures in the brain, such as the prefrontal cortex, amygdala and hippocampus. These limbic circuits harbour structures involved in emotion (amygdala) and planning (prefrontal cortex). In the hippocampus these emotional and cognitive events are placed in a context of place and time. Behavioural processes involve attention and appraisal, while also autonomic and neuroendocrine processes are orchestrated through the paraventricular nucleus of the hypothalamus (PVN) [42, 78]. The PVN is the central assembly point of the neural afferents mediating the stress response. Here all the "threatening" information converging from different brain areas, either directly or indirectly, ultimately activates the HPA axis [77, 95, 123].

The PVN consists of a magnocellular and parvocellular part. Magnocellular neurons produce vasopressin (AVP), which is released at the posterior pituitary into the blood circulation [141, 143]

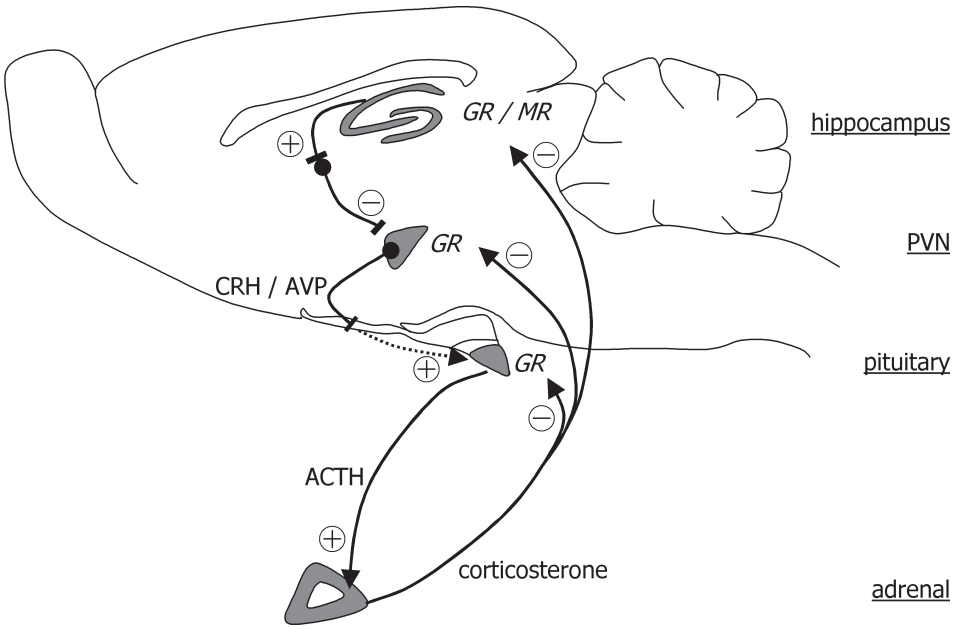

Figure 1.1
Schematic overview of the involvement of the hippocampus and hypothalamic-pituitary-adrenal axis (HPA axis) in the neuroendocrine stress response in rodents. Activation of the paraventriculuar nucleus (PVN) of the hypothalamus from brain stam and higher brain areas initiates the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the median eminence. At the pituitary CRH and AVP act in synergy to release adrenocorticotropic hormone (ACTH) into the blood stream. ACHT stimulates the adrenal cortex to secret corticosterone, which, in turn binds to the brain glucocorticoid (GR) and mineralocorticoid (MR) receptors to control the activity of the HPA axis via negative feedback mechanisms and thereby restores homeostasis. For a more detailed description of the cascade of events see ***Sections 1.1*** and ***1.2***.

to regulate water reabsorption by concentrating urine in the kidneys [18, 142, 201]. Parvocellular neurons produce besides AVP also corticotropin-releasing hormone (CRH). Both hormones are, upon stimulation, released from vesicles into the portal blood system at the median eminence. The portal blood system forms a direct link between the median eminence and the anterior pituitary. Upon reaching the pituitary CRH and AVP bind to their respective receptors, stimulating the production and release of adrenocorticotropic hormone (ACTH) into the blood stream. Although CRH is considered to be the main activator of ACTH, its effects are amplified by AVP [140, 153]. ACTH then travels via the blood stream to the adrenals, where it binds to its melanocortin (MC2) receptor located in the adrenal cortex [22]. Here, ACTH stimulates the synthesis and secretion of glucocorticoids in the circulation, *i.e.* corticosterone in rodents and cortisol as main product in man. The main effects of glucocorticoids on the brain are modulation of their own secretion (negative feedback) [36, 95], modification of neuronal integrity and function [62, 86, 122] and modulation of memory and learning processes [40, 42, 119]. All these processes ultimately serve to restore homeostasis at the level of behaviour and physiology. A more detailed description on glucocorticoid functions and effects is presented in the next section (***section 1.1.2***).

1.1.2 Glucocorticoid functions and effects

This section describes glucocorticoid functions and effects as observed in adult rodents. During early development not every component is fully developed yet, which can influence these

glucocorticoid-mediated effects. This aspect of glucocorticoid function will be discussed in more detail in ***section 1.3***.

Since almost every cell type in an organism is sensitive to glucocorticoids, these molecules have a wide range of actions. When plasma levels of glucocorticoids rise in response to stress they stimulate catabolism, mobilise lipid and glucose reserves, suppress immune responses and increase cardiovascular tone [132, 133]. Glucocorticoids also affect emotional responses and cognitive processes. Hence, they are necessary for behavioural adaptation to any stressful situation. For example, increased corticosterone directly following a learning task promotes consolidation of memory in rats and mice. These animals then "remember" the task and are better able to cope with the same situation a second time they encouter it. On the other hand, when this rise in corticosterone takes place "out-of-context", indicating that no direct relation to the task can be made, memory storage and retrieval of the task might even be impaired [40, 87, 137].

In rodents, the active glucocorticoid is corticosterone. In humans two glucocorticoids are secreted, cortisol and corticosterone. Cortisol is believed to be the main glucocorticoid based on its 10 to 20 times higher concentrations present in blood [89, 186]. However, whether the central effects of glucocorticoids in humans are also dominated by cortisol remains to be investigated. Karssen *et al.* [92] showed that cortisol is selectively hampered from passing the blood-brain barrier by a multi-drug resistance protein called P-glycoprotein, which is likely to result in (only) a 6-fold higher concentration of cortisol compared to corticosterone in the brain. The lack of P-glycoprotein-mediated transport of corticosterone in contrast to cortisol suggests a more important role for corticosterone in modulating human brain function than previously recognised [92] and increases the validity of investigating corticosterone effects in regulation of the stress response in rodent models.

Although increased glucocorticoid levels in response to stress are beneficial to an organism, exposure to high levels for a longer time can also have deleterious effects and lead, for example, to atrophy of apical dendrites in the hippocampus [56, 62, 122]. To prevent the production of excessive amounts of corticosterone by the HPA axis, glucocorticoids produce a negative feedback control on various levels in the cascade of a stress response, thereby reducing their own secretion.

In order for glucocorticoids to exert their effect they bind to two receptors: the mineralocorticoid and glucocorticoid receptor (MR and GR, respectively) [38, 126]. Both MR and GR are cytosolic receptors, which are transported to the nucleus upon binding to their ligand [42]. In the nucleus they act as transcription factors and modify gene expression in various ways. In the form of homodimers MR and GR can bind to glucocorticoid responsive elements located in the genome and hence cause transactivation or repression of genes [11, 46, 185]. MR and GR can also interact, as monomers, with other transcription factors in a complex of protein-protein interactions and thus affect gene expression in a process that is called transrepression [60].

Both MR and GR are expressed throughout the body and the brain, but differ in their

distribution and affinities for corticosterone [38, 151]. Though MR is expressed throughout the body [100], its expression in the brain is predominantly located in limbic regions, like the hippocampus [177, 189]. In epithelial cells, such as in the kidney, the ligand for MR is aldosterone, which becomes corticosterone if inactivated by 11ß-hydroxysteroid dehydrogenase (11ß-HSD). Non-epithelial cells in the heart and brain contain a non-selective MR predominantly occupied by circulating corticosterone. The GR, on the other hand, is bound by glucocorticoids both in the body and in the brain [42]. Central GR has a more widespread expression pattern in the brain than MR. Particularly high expression is found in the hippocampus, PVN, hypothalamic nuclei, the cortex, amygdala and brainstem, all regions involved in regulation of the behavioural and endocrine stress response [177, 189].

Even though both central MR and GR can bind glucocorticoids [38, 126, 197], their pharmacological properties are quite different. MR has a 10-fold higher affinity for corticosterone than GR [151]. As a consequence, the MR is already occupied at basal circulating corticosterone levels regulating the tonic activity of the HPA axis (proactive mode). Additional GR occupation at the circadian peak or after a stress response is essential for the HPA axis to return to homeostasis (reactive mode) [41, 42]. For an appropriate function of MR and GR in, for example, the hippocampus the balance in actions mediated by these two receptors is critical and important for neuronal excitability, stress responsiveness and behavioural adaptation. A deregulation of this balance thus increases vulnerability for psychiatric disorders [42].

1.2 Adverse early life events in humans

1.2.1 Association between glucocorticoids and depression

Many studies nowadays point to the involvement of the stress system in the aetiology of affective psychopathology [12, 13, 27, 58, 69, 71, 72, 82, 152]. For example, an estimated 50% of patients suffering from major depressive disorder displays a dysfunctional HPA axis [152]. The main characteristics are increased cortisol levels (blood plasma, urine and cerebrospinal fluid), an increased cortisol response to ACTH, blunted circadian rhythm and impairment of cortisol suppression after dexamethasone treatment [82]. One of the most compelling pieces of evidence describing a relation between depressive symptoms and stress is the observation that deregulation of the HPA axis even precedes the onset of depression. Furthermore, the relieve of these depressive symptoms are preceded by a normalisation of the HPA axis [82].

Belanoff and colleagues, who successfully treated patients suffering from psychotic depressive disorder with GR antagonists, have recently provided further proof of the close relation between depressive mood disorders and the HPA axis. They showed that short-term use of a high dose of GR antagonist reduced psychotic and depressive symptoms by 50% [13].

One of the clearest functional relations between psychotic depression and HPA axis functioning is revealed by research on Cushing's syndrome. Cushing's syndrome is caused by an adrenal or pituitary adenoma and is besides high blood pressure, hyperglycaemia, hypokalaemia

and obesity, in a significant number of patients also characterised by an impaired memory function, insomnia, anxiety and psychotic depression. Since a pituitary or adrenal adenoma causes excessive levels of cortisol in the body (hypercortisolemia), GR antagonists were used to normalise the physical parameters mentioned above, influenced by these high cortisol levels. Strikingly, this treatment also revealed that the observed neuropsychological symptoms were directly correlated with circulating cortisol levels that were resolved quickly when the hypercortisolemia was treated [152, 166].

1.2.2 Epidemiology of depression

Depression is a widespread mental disorder occurring regardless of gender, age or ethnical background. It is characterised by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep and/or appetite, low energy and a poor concentration [1] and leads to substantial impairments in an individual's ability to take care of his or her everyday responsibilities. Major depressive disorder is characterised by a severely depressed mood that persists for at least two weeks and may be specified as either "a single episode" or "recurrent". At its worst depression can lead to suicide, associated with the loss of about 850 000 lives every year worldwide [205] and affects about 16% of the population on at least one occasion in their lives [15]. Almost without exception, epidemiological studies have documented higher rates of depression in women than in men (mean prevalence of 7.3% versus 4.0%) [15].

The World Health Organisation ranked depression in 2000 as the leading cause of disability in the US as well as in other countries and as the fourth leading contributor to the global burden of disease, which is classified as "Disability Adjusted Life Years" (DALYs; the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability). Nowadays, depression is already the second cause of DALYs in the age category 15 to 44 years and by the year 2020 depression is expected to reach the second place after heart disease of the ranking of DALYs calculated for all ages and both sexes [134].

1.2.3 Genotype - environment interactions in the onset of depression

The onset and persistence of depressive episodes can be influenced by genetic, biological and psychosocial factors (see ***Figure 1.2***). To examplify the importance of psychosocial factors, such as adverse living conditions, 18% of preadolescents and 40% of adolescents with a history of child maltreatment meets the current diagnostic criteria for major depressive disorder [51, 127]. The consistent finding that many individuals with a depressive disorder exhibit an abnormal functioning of the stress system has led to the development of animal models that emphasise the role of development, environment and genotype in shaping pathways susceptible to affective illness [28]. In particular exposure to prenatal or early postnatal stress, like under-nutrition during pregnancy, postnatal separation of the infant from the parents and sexual or physical abuse during childhood may interact with an individual's genetic predisposition and thus increase the risk of developing depression or any other mood or anxiety disorder [48, 69, 73, 131].

A relatively common observation in psychiatry is the association of major depressive disorder with elevated levels of plasma cortisol in these patients [59, 67]. Since activity of the HPA axis can be affected by exposure to repeated and/or chronic stressors in ways hypothesised to contribute to mood disorders [157], early adverse experiences are believed to modify programming of the brain [74]. Early life stress has been associated with (juvenile) onset of major depressive disorder [85, 96] with a linear dose response relation between the severity of abuse and risk of depressive episodes [204]. Early life stress is furthermore associated with persistent hyperactivity of the HPA axis and the autonomous nervous system as well as with increased sensitivity of these systems in adult patients [27, 71, 72]. The observation that neglect in childhood can suppress growth of the long bones is another example of how an early experience can have a tremendous impact on development [4, 57, 175]. This may in part reflect the effect of chronic stress operating via the HPA axis, as CRH and glucocorticoids are known to influence the growth hormone system and

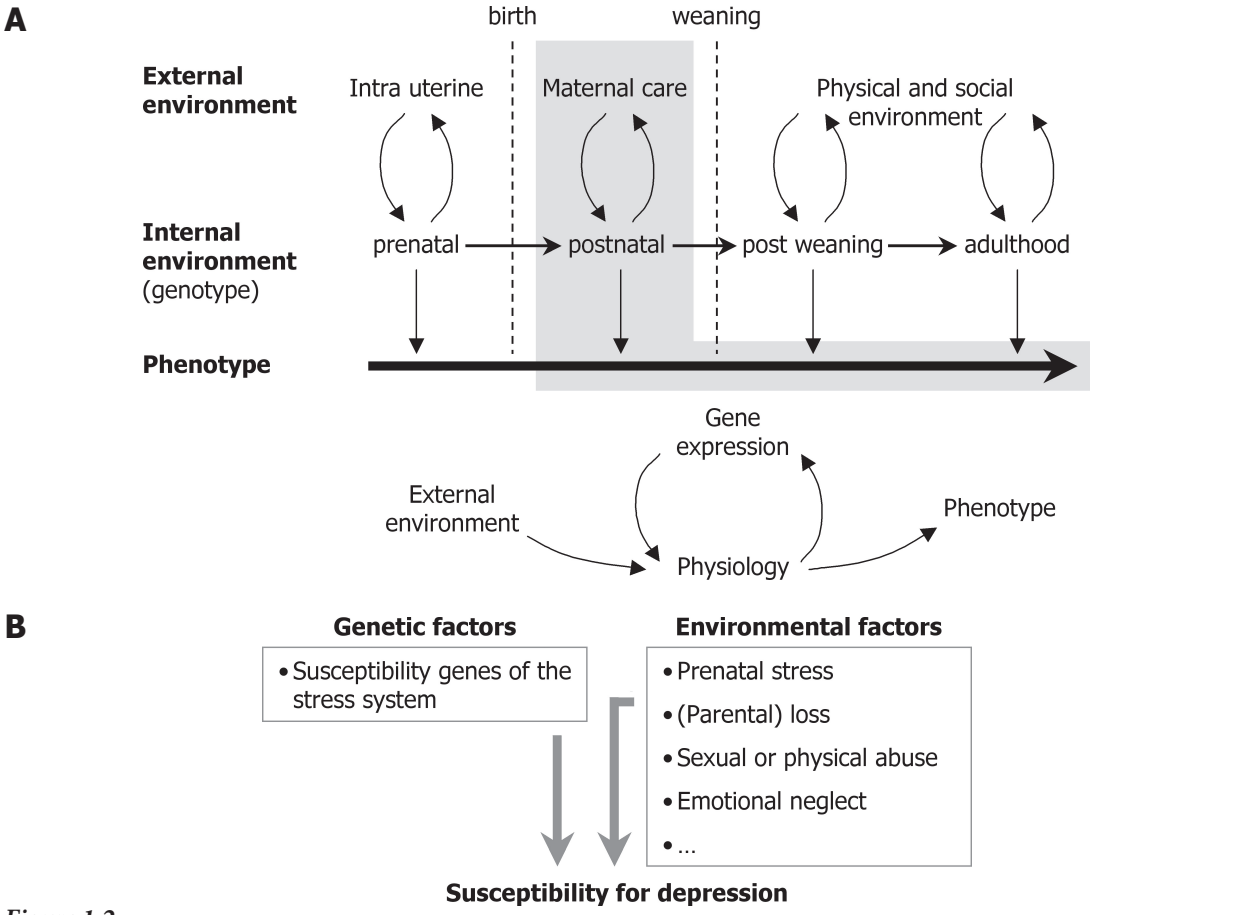

Figure 1.2
(***A***) A schematic overview of the interactions between the genome and environment shaping the phenotype of an organism throughout its life. The focus of this thesis is on early postnatal period between birth and weaning in mice (gray area). Variations in maternal care or physiological changes in the pup, whether experimentally induced or naturally occurring, influence long-term development of structure and function of both physiological and behavioural characteristics. Disruption of infant's homeostasis by atypical maternal care represents a critical period of sensitivity of these interactions and is used in this thesis as an animal model to study the consequences of traumatic early life events. The design of the figure is based on a scheme kindly provided by Christopher R. Price, with minor modifications [150].
(***B***) Some examples of environmental factors that increase the susceptibility for developing depressive disorders as acquired from human epidemiological studies.

the production of growth factors in ways that suppress growth [67, 88].

Results from animal studies also indicated that stress early in life can promote long-term changes in multiple neurotransmitter systems and brain structures involved in the aetiology of major depressive disorders in adults [8, 68, 74, 94]. It is therefore hypothesised that neurobiological changes associated with adverse early experiences can confer vulnerability for the development of depression [93].

1.3 Characteristics of normal HPA axis development

In psychobiology it has long been recognised that postnatal parental care is an important environmental regulatory factor for an individual's development [80]. Human epidemiological and animal experimental studies show that early social experiences influence the functioning of physiological processes even into adulthood [70, 80, 145]. As noted in ***section 1.2.3*** and illustrated in ***Figure 1.2.B***, early exposure to adverse experiences increases the risk for development of, among others, posttraumatic stress, depression and anxiety disorders [70]. However, to understand the molecular mechanisms underlying the effects of adverse early life events and the effects on HPA axis functioning it is eminent to have knowledge on the normal development of the stress system and factors that regulate this development. In the following sections the main issues related to development of the stress system in rodents, important for the understanding of the research described in this thesis, will be discussed.

1.3.1 Stress hypo-responsive period

The stress hypo-responsive period (SHRP) lasts from postnatal day (pnd) 4 to 14 in rats [112, 113, 167, 200, 207] and from pnd 1 to 12 in mice [169]. Very low circulating basal levels of ACTH and corticosterone characterise this period. In response to most mild stimuli, like isolation, novelty or saline injection, pups do not show an activation of the pituitary-adrenal axis like their older conspecifics and are therefore called hypo-responsive. Additionally, these pups have no circadian rhythmicity in ACTH and corticosterone secretion. For rat pups it was shown that they do not display the characteristic early evening peak, corresponding to the start of the active period and daytime trough of these hormones until they are 21 to 25 days old [2].

In a recent study it was furthermore demonstrated that in early postnatal development of the HPA axis mice show a high expression of CRH mRNA in the PVN [169] (see ***Figure 1.3***). This corresponds to earlier data published for both mice and rats [44, 45, 172]. Expression levels of GR mRNA in the hippocampus were only measurable for the CA1 area, since other areas remained below the detection limit. Expression in this area was low at birth, but increased significantly during the SHRP reaching highest expression at pnd 12. Earlier reports on the ontogeny of GR mRNA expression in the rat also showed a gradual increase with age [17, 130, 161, 165], though most of these studies examined the regional ontogeny in detail. Interestingly, one study reported that GR immuno-reactivity in the CA3 region of the hippocampus was only clearly present

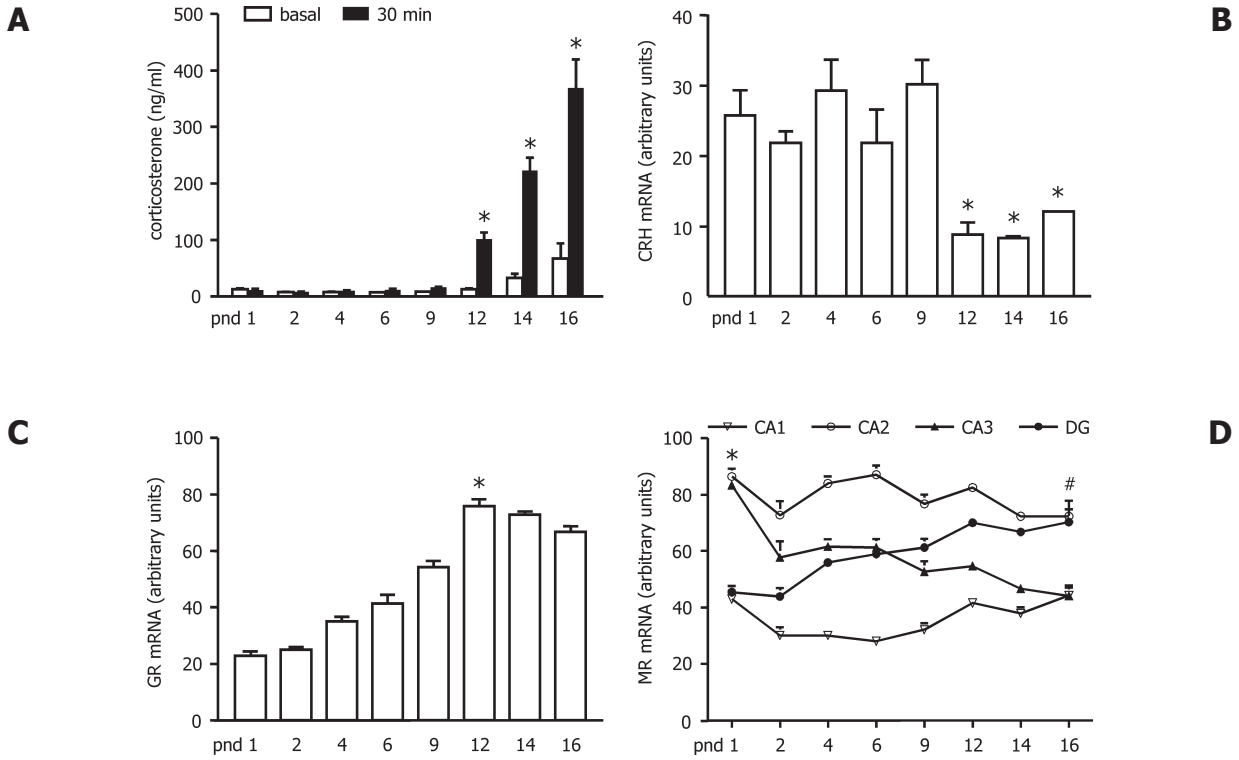

Figure 1.3
Four sample figures illustrating the dynamic developmental changes occurring in various HPA axis markers in CD1 mouse pups at different postnatal ages. (***A***) Basal plasma corticosterone concentrations are low from pnd 1 to 12 and increase from pnd 12 onwards. Between pnd 1 and 9 there is no increase in corticosterone secretion following novelty stress. In contrast, pups at pnds 12, 14 and 16 do respond to 30 minutes of novelty with increasing elevations of corticosterone. * $P<0.05$ versus basal. (***B***) Basal expression of CRH mRNA in the PVN is high at birth (pnd 1) and shows a sudden drop in expression at pnd 12. * $P<0.05$ versus pnd 1-9. (***C***) Basal expression of GR mRNA in the CA1 area of the hippocampus is low at birth (pnd 1), starts to increase from pnd 4 onwards and reaches highest expression at pnd 12. * $P<0.05$ versus pnd 1-9. (***D***) Basal expression of MR mRNA in the CA1, CA2, CA3 and dentate gyrus (DG) area of the hippocampus. MR mRNA expression is higher in the CA1 and CA3 area compared to CA2 and DG at birth. During further development expression remains constant for CA1 and CA2, while expression levels for CA3 and DG reverse. * $P<0.05$ versus CA1 or DG at pnd 1, $^{\#}P<0.05$ versus CA1 or CA3 at pnd 16. Adapted from Schmidt *et al.* [159].

during the first week of life in rats and then disappeared [161]. When mice had developed past the SHRP they exhibited enhanced corticosterone basal levels and a response of both ACTH and corticosterone to mild novelty stress. CRH mRNA expression decreased significantly, while expression of GR in the CA1 area of the hippocampus remained high with a small decrease at pnd 16. In mice the expression of MR in the hippocampus was as high as in adults throughout the postnatal development of the HPA axis and changed very dynamically in a time- and subregion-specific manner [169].

1.3.2 Maintaining HPA axis hypo-responsiveness in early development

During the SHRP the HPA axis differs in a number of functions relative to the adult rodent, resulting in an inhibition of peripheral stress responses. Several intrinsic factors have been identified that are (partly) involved in maintaining the pituitary-adrenal hypo-responsivity. The adrenal cortex is relatively insensitive to ACTH stimulation, releasing only a minimal amount of corticosterone in response to exogenous ACTH stimulation [159, 178]. Furthermore, while a

mild stressor is able to rapidly activate CRH mRNA expression during the SHRP [45], this does not result in increased circulating ACTH. This indicates an inefficient CRH release or signal transduction to the pituitary. Possibly corticosterone itself plays a role in the maintenance of the SHRP as well. During the SHRP the concentration of corticosteroid-binding protein, which binds approximately 75% of the glucocorticoids in the circulation of adult rats, is very low and results in relatively high levels of biologically active corticosterone in the blood [75]. Though the total corticosterone concentration is low, the levels of free corticosterone could be sufficient to exert enhanced feedback at several levels of the HPA axis during the SHRP (see also ***Figure 1.4***). The pituitary has been identified as the main site of glucocorticoid feedback action in rat pups, partly due to its adult-like levels of GR mRNA expression [163]. The PVN as well as an altered balance between MR and GR in the hippocampus which projects to the PVN, might also contribute to the suppression of the HPA axis during this hypo-responsive period [169, 209].

Maintaining minimal corticosterone in plasma is hypothesised to prevent harmful effects of either too high or too low hormone concentrations on the developing nervous system. High levels of glucocorticoids are known to cause structural changes in pyramidal neurons of the hippocampus, *i.e.* dendritic atrophy and an impairment of neurogenesis in dentate gyrus neurons [55, 56, 61]. These effects, though, were shown to be reversible when normal glucocorticoid levels were reinstated. A total absence of corticosteroids, on the other hand, leads to apoptosis [42]. Neonatal treatment of rat pups with glucocorticoids affected the pattern and reduced the rate of postnatal granule cell genesis in the hippocampus [16]. Furthermore, neonatal exposure to hydrocortisone decreased HPA axis responsivity to stress at weaning (approximately pnd 21) and an impairment of the adrenocortical response to stress at 45 to 48 days of age [49]. Furthermore, after a single 24 hours maternal deprivation increased cell death of neurons and glia in several

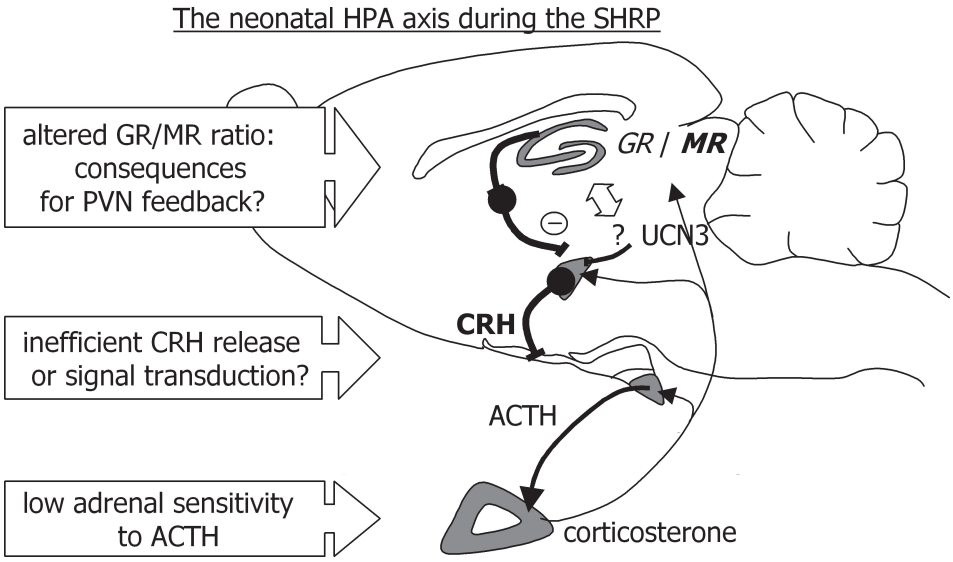

Figure 1.4
Schematic illustration of the stress system during the SHRP indicating the significantly altered state and function of the HPA axis at different organisational levels as compared to the adult. See ***Figure 1.1*** for abbreviations and an explanation of the adult stress response. Adapted from Schmidt *et al.* [159].

cortex regions was observed when the deprivation was performed within the SHRP, but not when performed outside the SHRP [210]. Consequently, disturbances of normal HPA axis development during the SHRP are expected to result in long-lasting alterations in neuroendocrine functioning and behaviour.

1.3.3 Maternal influence on rodent development

Apart from intrinsic factors (see ***section 1.3.2***) maternal factors also contribute to the pituitary-adrenal hypo-responsivity during early development [39, 113]. The mother is the only providing source for many features essential for pup survival. She provides food (milk), warmth (temperature regulation) and active interaction (licking and grooming) [80, 103, 158] and rodent mothers rarely leave their offspring for more than 15 to 20 minutes [3]. Experimental manipulation of the mother-pup interaction (*i.e.* maternal separation) provides clues on the role of maternal influences on pup development. Already after several hours of maternal absence, basal and stress-induced ACTH release are enhanced, followed by an increased corticosterone secretion from sensitised adrenals [102, 160, 170, 178]. Furthermore, CRH and GR mRNA expression in the PVN and MR and GR mRNA expression in the hippocampus decreased [170, 172]. The expression of *c-fos*, an immediate early gene used as marker for neuronal activation, showed a 100-fold increase in the PVN [176]. Interestingly, when mimicking part of maternal behaviour by stroking the anogenital region of rat pups for 30 to 45 seconds every 8 hours, the rise in ACTH and decrease in CRH and MR mRNA expression could be prevented [180]. Strikingly, even the *c-fos* activation was then abolished [192, 194]. Feeding and stroking restored corticosterone and GR mRNA levels as well, indicating that starvation increases the adrenal sensitivity for ACTH [192, 194].

Recently, Schmidt *et al.* [171] provided further evidence that the activation of the HPA axis due to prolonged maternal absence during the SHRP may be closely related to metabolic responses. As in adults, food deprivation in pups leads to a decrease in blood plasma glucose and leptin and an increase in plasma ghrelin levels [19, 25, 90, 91, 97, 99, 208]. High levels of glucose suppress ghrelin release from the stomach [91, 99] and therefore increasing ghrelin levels are a direct reflection of decreasing glucose levels. These responses are further substantiated by the observed ACTH response to 24 hours of maternal absence [170], which is also observed for food deprivation of adult rats [35]. By blocking the metabolic signals of glucose (by applying a high dose of glucose) and ghrelin (by applying a ghrelin antagonist) during 8 hours of maternal separation, Schmidt *et al.* were able to (partially) prevent the pituitary-adrenal activation normally associated with prolonged maternal separation [171]. Concomitantly, this treatment prevented changes in expression of neuropeptide Y (NPY) mRNA in the arcuate nucleus and CRH mRNA in the PVN indicating the importance of these signals in the activation of the HPA axis during the SHRP. Leptin treatment, however, did not affect separation induced effects on the HPA axis [171] (see also ***Figure 1.5***).

Further evidence for maternal influence on rodent development comes from studies using inter-individual differences in licking and grooming (LG) and arched back nursing (ABN)

displayed by rat mothers of the same strain during the first two weeks after delivery. Mothers exhibiting high levels of LG-ABN have offspring that is less fearful and shows a modest HPA axis response to stress compared to offspring from low LG-ABN mothers [52, 118, 203]. When pups from low LG-ABN mothers are then cross-fostered to high LG-ABN mothers, the adult offspring will display the phenotype of the high LG-ABN mothers and *vice versa*, when pups from high LG-ABN mothers are cross-fostered to low LG-ABN mothers, the adult rats will display the phenotype of the low LG-ABN mothers, both on molecular characteristics as in behaviour [52, 203]. These studies clearly show that maternal behaviour plays a very important role in HPA axis development in rodents and that this maternal behaviour (within a strain) is learned.

Apart from inter-individual variation of maternal behaviour within strains, effects as a consequence of the genetic background are also known. BALB/c mice, which are characterised to be very fearful, as illustrated by their elevated endocrine responses to stress, become less fearful when raised by C57BL/6J mothers [7]. The higher licking and grooming frequency of

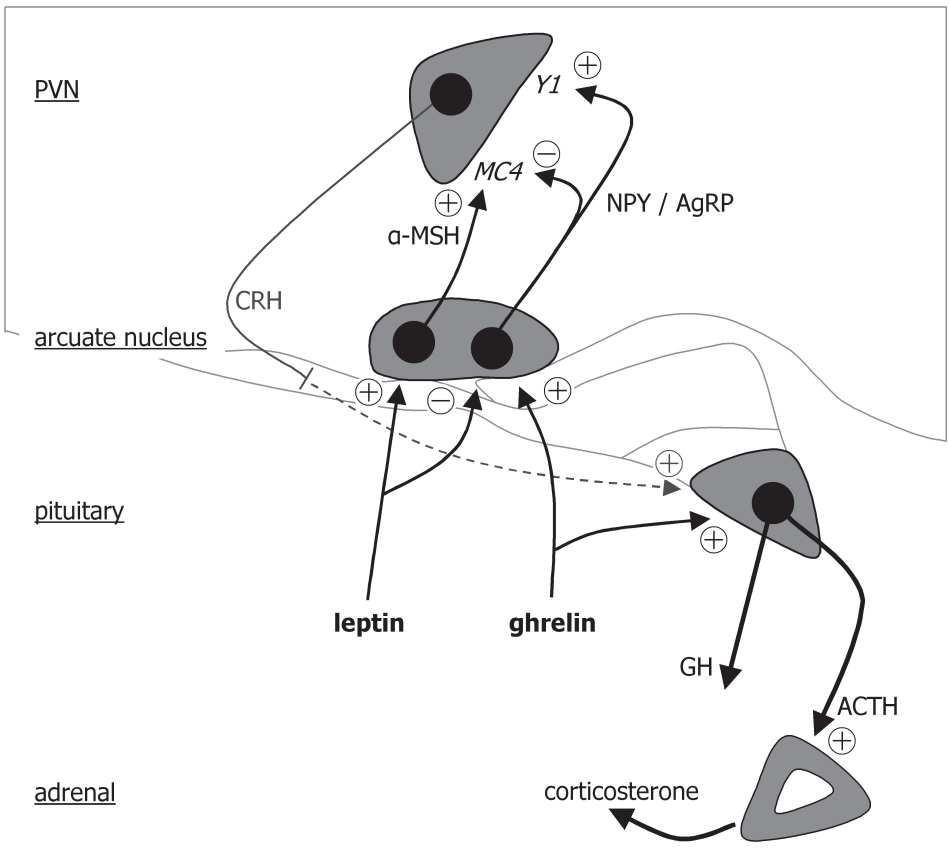

Figure 1.5
The dynamics in the influence of metabolic signals leptin and ghrelin on HPA axis functioning. Ghrelin is synthesised in the stomach and reaches the arcuate nucleus of the hypothalamus via the blood stream. Here ghrelin stimulates GABA-ergic neurons to produce and release neuropeptide Y (NPY) and agouti-related protein (AgRP). Via the NPY receptor (*Y1*) in the paraventricular nucleus (PVN) of the hypothalamus NPY and AgRP stimulate CRH release. However, NPY and AgRP have inhibitory effects via the melanocortin 4 receptor (*MC4*) receptor. In the pituitary ghrelin functions as a releasing agent for, *e.g.* growth hormone (GH) and adrenocorticotropic hormone (ACTH).
Leptin inhibits the GABA-ergic neurons of the arcuate nucleus, but stimulates melanocortin neurons. Upon stimulation, these pro-opiomelanocortin (POMC) neurons produce and release α-MSH, which in its turn via MC4 stimulates CRH release from the PVN.

C57BL/6J mother compared to BALB/c mothers explained this reduced fearfulness. On the other hand, C57BL/6J pups raised by BALB/c mothers did not "acquire" the BALB/c phenotype, but remained indistinguishable from C57BL/6J pups raised by their own mothers. To make things more complex, C57BL/6J pups that were cross-fostered as embryos to BALB/c mothers and raised by BALB/c mothers did show the BALB/c phenotype [53]. These studies indicated that for gene-environment interactions both the pre- and postnatal environment interact and determine the adult behaviour of inbred mouse strains [30].

1.3.4 Analogy between rodents and humans

The validity of animal models used to investigate the consequences of adverse early life events on HPA axis functioning clearly depends on the parallels that can be drawn between rodent and human early development of the HPA axis. Unlike rodents, humans (and also Rhesus macaques) exhibit an early morning peak and an evening trough in corticosterone within a few weeks of birth [147]. Similar to the rodent, the human HPA axis functioning is not adult-like at birth and the circadian regulation continues to mature into the third year of life in humans. However, at this age the daytime production of cortisol is still not fully consistent with the mature pattern [202].

The human HPA axis is furthermore quite responsive to stressful situations roughly up to 3 months of age, but then becomes increasingly difficult to activate over the course of the first year [63, 66]. This shows close similarity with the rat development, which displays a responsive HPA axis during the first few days of life, but becomes hypo-responsive to stress from pnd 4 onwards [112, 167, 207]. Tentatively, the period between 6 and 12 months of age marks the transition into a human functional equivalent of the rodent SHRP [64, 66]. However, what appears to be a period of hypo-responsiveness may more correctly be characterised as a period of intense psychosocial buffering of the HPA axis. This buffering is "caused" by the presence of an adult with whom the child has formed a secure attachment relationship or with an unfamiliar substitute caregiver who shows sensitive and responsive behaviour towards the child [66, 135].

During early development in rodents and primates, contact with responsive, nurturing caregivers appears to be critical for the development of the neuroendocrine system. As noted before, researchers have speculated that adverse early rearing environments in humans will enhance vulnerability to behavioural disorders in part through disturbing the development of stress-sensitive neurobiological systems, including the HPA axis [37, 65, 74]. For infants and children neglect has already been shown to produce an apparent loss of daytime rhythm in corticosterone. Similar effects were demonstrated in nursery-reared Rhesus infants [20].

1.4 Adverse early life event modelling in rodents

Investigating the fundamental biological mechanisms that underlie the enhanced vulnerability to develop behavioural disorders in humans is troublesome. Apart from an ethical perspective,

both genetic and environmental factors that may induce the disorder cannot be controlled and are difficult to quantify. Furthermore, subjects cannot be randomised for treatment and studies may take decades to complete. Since an infant has numerous, changing neuroendocrine interactions during development, it is also impossible to model this accurate in *in vitro* conditions. Much of the current knowledge on human biology originates largely from research in experimental animals. Therefore, animal studies in which it is possible to control in a laboratory, at least to a certain extent, for environmental influences are essential to study behaviour in combination with a neurochemical analysis of brain function and hormone balance [31, 199].

1.4.1 Pre- and postnatal manipulations

The objective of this thesis deals with the influence of early life experiences on the development of the stress system (***section 1.5***). For this purpose, we chose to intervene in the normal development of a mouse by applying an experimentally-induced stress factor. Experimental intervention can be performed either pre- or postnatally.

Using prenatal stress, pregnant rodents are subjected to, for instance, crowding [34], noise [54], saline injections [144] or restraint stress [120]. This prenatal restraint stress does indeed affect adult offspring's behaviour, resulting in increased anxiety [188] and enhanced age-related recognition memory impairment [187]. On the other hand, also HPA axis responsiveness is altered, showing increased responsiveness [98, 121], reduced hippocampal MR and GR density [76, 121], impaired glucocorticoid feedback [121], an abolished SHRP [76] and accelerated the age-related HPA axis dysfunction [120, 187]. Furthermore, the hyperactivity of the offspring's HPA axis induced by prenatal stress is related to high levels of maternal corticosterone secretion during restraint stress [10, 120]. Interestingly, also maternal behaviour towards the pups after birth has been shown to be affected by prenatal stress. Adoption of prenatally stressed pups reared by undisturbed dams prevented the impairments in glucocorticoid feedback [121].

Since adverse experiences early in human life are shown to affect normal development (see ***section 1.2.3***), also postnatal animal models have emerged to investigate causes and consequences using the separation of mother and pups in rodents and primates to experimentally introduce such an adverse life event. This separation is a stressful situation as maternal care plays a major role in the postnatal development. Besides food, the mother also provides thermal, somatosensory, kinaesthetic, olfactory, visual and auditory stimulation. Already after short periods of separation rat pups show a strong ultrasonic vocalisation [81]. When the separation is prolonged their heart rate declines, sleep/wake cycles get disturbed and growth is affected [79, 101]. All these observations indicate that the maternal environment is capable of influencing the postnatal development (see also ***section 1.3.3***). Indeed, these effects on offspring's behaviour and neuroendocrinology have been reported and are described in detail in ***sections 1.4.3*** to ***1.4.6***.

The most often applied postnatal manipulation models are "(early) handling", involving daily separations of mother and infants for up to 15 minutes [32, 33, 111, 115, 117, 198] and the "repeated separations" paradigm that comprises of repeated separations of the pups from the

mothers for a period of 3 to 6 hours per day [104, 105, 107, 117, 145, 146, 155, 156] (see also ***Figure 1.6***). Both paradigms are applied during the SHRP or during the period from birth until weaning. Generally, the consequences of handling are considered to be beneficial for the development of the HPA axis. In contrast, repeated separations are considered to be detrimental and result in increased pituitary-adrenal responses to stress in adult animals and in a decreased negative feedback [117, 145]. A third model to simulate an adverse early life event in rodents is "maternal deprivation", which consists of the separation of mother and pups for a single period of 24 hours at some stage during the SHRP [39, 179, 181, 184, 192, 194, 206, 207] (see also ***Figure 1.6***). In this thesis, both the "repeated separations" and the single 24 hours "maternal deprivation" model are used as an experimentally-induced adverse early life event to investigate the consequences for HPA axis development in mice.

Pre- or postnatally, any experimental paradigm applied in rodents is a model to gain knowledge about stress system development to eventually draw parallels for the understanding of human brain development. The corresponding critical time period between humans and mice, in which to (experimentally) intervene in normal development can be addressed from different view points. Judging the mouse pup by its state of brain development, *i.e.* neuronal growth and neuron connectivity, this period corresponds to the last trimester of human pregnancy [6]. However, comparing the functional state of the HPA axis, resemblance is shown to a 6-12 months old human infant [64, 66]. This clearly shows that, though there are similarities, there also remain differences between rodent and humans. For the studies described in this thesis the choice was made to use a postnatal manipulation paradigm. Not only because there is already a large amount of data on both short- and long-term effects of a traumatic early life events, but the prospect of possible pharmacological interventions when the functional state of the HPA axis is altered due to an early life experience also provides a wealth of experimental options not yet possible with prenatal manipulations.

As indicated in ***section 1.1.1***, several brain structures are involved in or affected by an HPA axis response to stress. Many systems are affected by changes in the early environment of rodent pups, but also of primate infants, which are more closely related to humans. These effects are described in reviews by, for example, Pryce [149, 150], Sanchez [164], Ladd [104], Suomi [182, 183] and Roman [154]. Since this thesis mainly focuses on the interaction of HPA axis development and the early life environment, the effects of adverse early life events on the HPA axis will be highlighted in the description of the short and long-term effects of the two applied models, *i.e.* repeated separations and maternal deprivation.

1.4.2. Methodological considerations

The choice of the control group in studies investigating the role of environmental factors on development proved to be very complex. In many studies manipulated rats are compared to rats that do not experience any direct human environmental disturbance during infancy ("non-

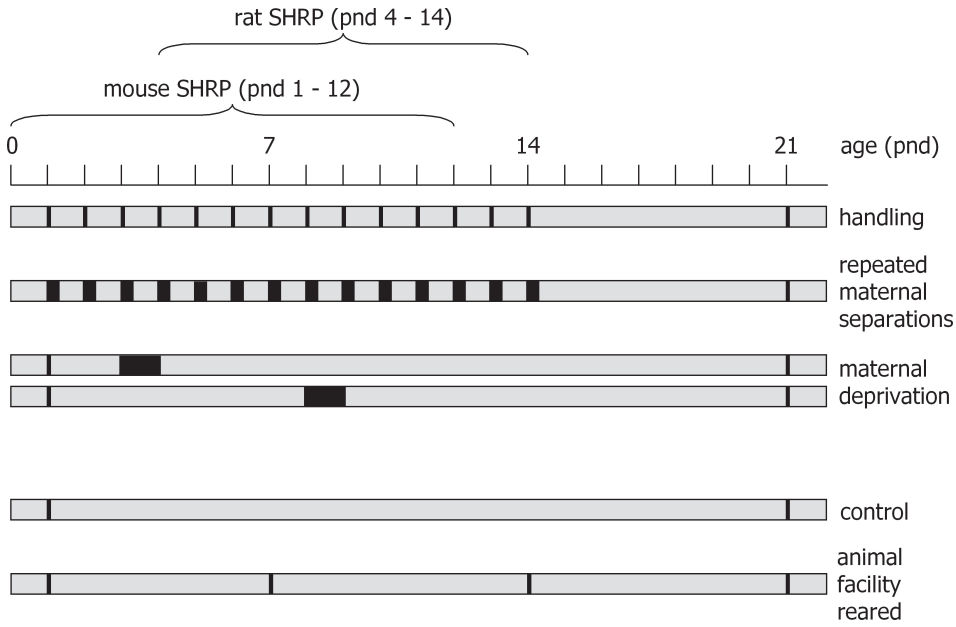

Figure 1.6
Illustration of the various types of maternal separation paradigms that are used. The top line indicates the age of the rodent in postnatal days (pnd). The day of birth is indicated as pnd 0. Above, the stress hypo-responsive period of the mouse (pnd 1 to 12) and the rat (pnd 4 to 14) is marked by large brackets. Usually, at pnd 1 the number of male and female pups is determined and reduced to a fixed litter size with an equal number of male and female pups (culling). Gray bars show different maternal separation/deprivation paradigms. The first bar represents a typical "handling" paradigm: rodent pups are separated from their mother daily for short period of time, usually 3 to 15 minutes. The second bar represents a typical "repeated maternal separations" paradigm: pups are separated from their mother daily for longer periods of time, usually 3 to 6 hours. The third and fourth bar indicate a single 24 hours "maternal deprivation", which can be performed at any stage during early development (as an example pnd 3 and pnd 8 are indicated). The developmental stage at which maternal separation is performed determines the short- and long-term consequences.
The two lowest bars present the most frequently used control groups in literature. "control": pups experience the standard procedures for culling and determining sex ratio's in the nest, but then remain undisturbed until weaning. "animal facility reared" is treated like "control", but the pups also experience the human interventions inherent to standard cage cleaning in animal facility husbandry.

handling") [108, 118, 124, 145, 148], while in other studies control litters experience the lab's routine husbandry regimen, including occasional brief handling for cage cleaning ("animal facility reared") [108, 148] (see also ***Figure 1.6***). Depending on the control group used repeated separations result in, for example, a hyper-reactive, hypo-reactive or non-affected HPA axis (for a detailed analysis see review [149]). In addition, any (experimental) manipulation will also affect maternal behaviour, so the relative effects observed in the treatment group might be diluted or exaggerated due to an altered maternal behaviour in the control group [150]. The effects of these manipulations might lead to increased maternal care following reunion, disturbance of pup homeostasis related to prolonged absence of the mother or a combination of these factors. To complicate this issue even further, the adult phenotype might also be affected by the maternal physiology during lactation (*i.e.* a stress-induced corticosterone increase in the dam, which is transmitted via the milk), resulting in lifelong changes in neuroendocrine functioning and behaviour [26, 150]. From the above it is clear that a careful selection of the control groups is therefore of eminent importance and should always be taken into consideration when comparing results from varies studies.

The best way to control for effects on neonate development induced by maternal behaviour would be the observation of maternal care for 24 hours from birth until weaning. This, however, is barely feasible. Since also nest size [158] and the ratio of male and female pups [5, 158] affects maternal behaviour, keeping these two factors constant for all the nests within and between experiments is workable. Furthermore, the pups used in each experimental treatment group should come from a number of different nests to control for inter-litter variation. Of course, also a clear description of the materials and methods is required.

1.4.3 Repeated maternal separations – 'short-term' effects

Very little data is published on acute (short-term) effects of repeated maternal separations on neuroendocrine development in rodents. The studies that do present observations on the acute results show mainly data on ACTH and corticosterone. Repeated maternal separations of rat pups for 1 hour per day on pnds 2 to 9 resulted in increased ACTH and corticosterone releases in response to the 1 hour maternal separation period at pnd 9 [124].

Also when pups were separated daily for a longer period of 3 hours, pups displayed elevated ACTH and corticosterone 5 minutes after the reunion with their mothers [83, 164]. Furthermore, when these pups were exposed to stress directly following the maternal separation period by pnd 14, like restraint or rat odour, they displayed an enhanced ACTH and corticosterone response when compared to pups that are either animal facility reared or handled [164].

The short-term changes induced by manipulation of the early environment of the pups are of major importance for the interpretation of data observed at later stages in life. It is hypothesised that these short-term changes modulate ongoing programming effects during development with long-term consequences. It is therefore crucial to take these short-term effects into account when the HPA axis development is studied in more detail.

1.4.4 Repeated maternal separations – 'long-term' effects

In contrast to 'short-term' effects, there is much more data published on the long-term effects of repeated maternal separations, although the results are rather inconsistent. The following paragraphs will describe the available data (including (some) conflicting findings) and a 'consensus' on the main findings.

Daily 3 hours maternal separation from pnd 2 to 14 is one of the best-characterised models with respect to long-term neuroendocrine changes in rats (age approximately 3 to 4 months). These separations lead to increased CRH mRNA expression in the PVN with a consequent increase in CRH peptide content in the median eminence. Surprisingly, basal and stress-induced plasma corticosterone levels are similar to non-handled rats, but are higher compared to handled rats [52, 145]. A 33% downregulation of CRH receptor binding in the anterior pituitary and concomitantly an increased noradrenergic response to stress might provide explanations for the increased CRH reactivity [52, 117]. Furthermore, these repeatedly separated animals show an upregulation of CRH gene expression in the bed nucleus of the stria terminalis (BNST) and central amygdala

(CeA), with a consequent increase in CRH peptide content in terminal fields of these neurons when compared to non-handled animals [129, 145].

The neuroendocrine responses to stress in adulthood seem to display varying results. Relative to animal facility reared controls, daily 3 hours maternally separated animals exhibit greater (2- to 3-fold) ACTH and corticosterone peak responses and more prolonged responses to air puff [104], restraint or novelty stress [128]. However, a prolonged, but not enhanced peak response of ACTH was observed by others of the same research group [104, 117]. Interestingly, rats that were repeatedly separated showed a 40 to 50% downregulation of hippocampal GR binding and mRNA levels as well as an upregulation of hippocampal MR binding and mRNA levels as adults. Also a decreased GR mRNA expression was observed in the medial prefrontal cortex [129]. Furthermore, these animals exhibited an altered GR functioning in the CA1 area of the hippocampus, discussed as a molecular basis for defective feedback regulation [14]. These data support the hypothesis of glucocorticoid feedback resistance at the hippocampus, suggesting an enhancement of HPA axis responsiveness to stress with enhanced and prolonged ACTH and corticosterone responses.

Apart from increased neuroendocrine responsiveness, repeated separations in both rats and mice also resulted in increased behavioural responses to stress [14, 52, 145]. Compared to handled controls these animals showed an increased anxiety-like behaviour in the elevated plus maze [84] and a higher emotional response in a light-dark box [14]. Interestingly, repeated maternal separations do not result in altered behavioural responses in, for example, exploration in open field, novelty-induced suppression of feeding, acoustic startle or a two-compartment exploratory task when these animals are compared to non-handled control animals [14, 24].

Despite conflicting data, which are most likely due to the various control groups used (see also ***section 1.4.2***), the overall consensus is that animals experiencing repeated maternal separations (3 to 6 hours) from pnds 2 to 14 display a fundamental reorganisation of neurocircuits involved in neuroendocrine regulation (PVN, hippocampus, frontal cortex), regulation of arousal and vigilance behaviour and autonomic nervous system tone as expressed in altered fear- or contextual-conditioning [164]. Therefore, repeated maternal separations is forwarded as a model to study the consequences of adverse early life events in humans [27].

1.4.5 Maternal deprivation – 'short-term' effects

Maternal deprivation procedures, most often for 24 hours, are used when the pups are still in the SHRP, which lasts from pnd 4 to 14 in rats [112, 113, 167, 200, 207] and from pnd 1 to 12 in mice [169] (***section 1.3.1***). Irrespective of the age within the stress hypo-responsive period, one of the most renowned effects of maternal deprivation is that it induces an increased ACTH and corticosterone secretion. Additionally, an endocrine stress response to a variety of mild stressors, like exposure to cold, novelty or saline injection, is observed when applied directly sequential to the maternal deprivation period [116]. Thus, the pituitary-adrenal axis of young animals is

activated and responsive to mild stressors, even though they are in the SHRP [178]. However, a single separation of mother and infant must last at least 8 to 24 hours to sensitise the adrenal cortex for a response to ACTH or novelty stress [116] to increase corticosterone secretion and to activate the sympatho-adrenal system [139].

Furthermore, the activity of the peripheral and central HPA axis is enhanced following maternal deprivation, as judged from the activation of *c-fos* and tyrosine hydroxylase mRNA expression in the adrenal and of *c-fos* and NGFI-B in the brain [139, 176]. These changes are accompanied by a downregulation of CRH and GR mRNA in the PVN and of GR and MR mRNA in the hippocampus [9, 170, 172, 196]. Also AVP mRNA expression in the PVN and $5HT_{1A}$ mRNA expression in the hippocampus are upregulated [21, 44, 47].

As mentioned in ***section 1.3.3***, maternal factors play an important role in HPA axis development. Mimicking properties of maternal behaviour has been shown to prevent some of the deprivation-induced effects [180, 192, 194]. There are, however, also intrinsic factors regulating the response to maternal deprivation. Recently it was shown that mice lacking the CRH receptor (CRHr1) in the pituitary did not show the characteristic increase in circulating plasma corticosterone and decrease in CRH and MR mRNA expression as is seen in response to maternal deprivation. This indicated that CRHr1 in the pituitary was essential in the initiation of maternal deprivation effects [173]. On the other hand, the absence of CRHr1 in the limbic system and forebrain resulted in a hyperactive corticosterone and ACTH response to maternal deprivation, whereas maternal deprivation was not able to downregulate MR mRNA expression in the hippocampus when CRHr1 in the limbic system was absent. This indicates a role for this receptor in mediating the suppression of the HPA axis [168].

The direct effects of maternal deprivation are relatively consistent across species and age at deprivation, provided the deprivation procedure is applied within the SHRP. When pups are deprived at a later age during the SHRP, the direction of the induced effects remains, but the amplitude increases due to maturing of the HPA axis [29, 116]. The longer-lasting, persistent effects on HPA axis activity, however, depend on the age of deprivation. Rats deprived at either pnd 3 or 11 are hyper- or hypo-responsive to stress at pnd 20 [190, 191]. Also maternal behaviour towards the pups varies with age and the absence of specific time-dependent maternal factors alters the HPA axis functioning both acutely and persistently [192]. Combined with a more mature state of the HPA axis of the pup, reactive negative feedback could restrain the HPA axis more effectively in older pups [169, 193]. All studies thus indicate that the age at deprivation might be a determining factor for the long-lasting consequences of maternal deprivation.

1.4.6 Maternal deprivation – 'long-term' effects

The long-term effects of maternal deprivation at pnd 3 resulted in an atypical pattern of corticosterone secretion during life. Where resting secretion of corticosterone in control animals hardly changed, deprived rats showed elevated levels of basal corticosterone secretion at young

age, dropped to bottom levels in the adult animal and slowly increased to reach levels comparable to control animals at senescence [206]. The responsiveness to novelty stress shows another age-dependent pattern. As adults (12 months old) maternally deprived rats were hyper-responsive compared to control rats, whereas both young (3 months old) and senescent rats (30-32 months old) were hypo-responsive [206].

When focussing on approximately pnd 50, pnd 3 deprived male rats then exhibit elevated basal ACTH and corticosterone and reduced hypothalamic and hippocampal MR and GR binding capacity, suggesting glucocorticoid feedback resistance [42, 184]. However, mRNA expression of both MR and GR were unaltered, although GR mRNA expression in the PVN and pituitary were reduced and hypothalamic CRH mRNA expression was reduced in the face of elevated ACTH [162]. Consequently, the actual pathway(s) leading to the adult phenotype thus remain poorly understood.

In a test on spatial learning and memory with these maternally deprived rats, the impairment in acquisition of information was observed at 3 and 12 months of age extending to disturbances in reversal learning compared to non-deprived litter mates. In addition, cognitive performance decreased with age. Surprisingly, at senescence there was no difference between the average performance of deprived and control animals, but the individual variation in cognitive performance increased dramatically. Most of the control animals were partially impaired, while the number of partially impaired animals of the deprived group was strongly diminished: they performed either excellent or were severely impaired. These findings suggest that maternal deprivation drives spatial learning abilities of senescent rats to the extremes at the expense of average performance [138].

The observation that not all rats behaved similarly underlines data from human studies, indicating that the consequences of adverse early life events vary per individual (see ***section 1.2.3***). Besides effects on learning abilities, this was also demonstrated for HPA axis regulation. Maternal deprivation at pnd 3 resulted in approximately 20% of adult rats in an HPA axis hyper-responsivity as compared to non-handled or animal facility reared control animals, when tested with a mild psychological stressor at postnatal day 90 [164]. Though very few studies investigated the long-term effects, the majority of the effects of this model seem to be confined to a several weeks period immediately following the date of 24 hours maternal deprivation [164].

1.4.7 Concluding remarks

On the basis of pre-clinical research with early adverse experience, maternally separated infant rats provide a model of depressive-like syndrome exhibited by *e.g.* a dysregulation of the HPA axis [24, 104, 106, 136]. This is, among others, shown by dexamethasone resistance [50, 104], enhanced anxiety-like behaviour and anhedonia [23, 104]. Data from the studies presented in ***section 1.2.3*** and ***sections 1.4.2*** to ***1.4.6*** indicate that the postnatal environment may modulate the neurobiological development of the infant, which thus has consequences for behaviour,

emotional responses and cognitive performance. The infant rodent is protected by the mother for most environmental influences, particularly if these concern relatively mild stimuli. For instance, exposure to a novel environment, which is a potent stressor in the adult animal, causes only minor activation of the HPA axis in the first two weeks of life [39, 112, 113, 167, 173, 207]. However, the infants HPA axis emerges from this hypo-responsive period when the mother is removed for a prolonged period of time. A pup deprived of maternal care for 24 hours shows enhanced basal activity and a profound HPA response when exposed to a novel environment [39, 113]. The impact of such an adverse experience in the developing infant furthermore depends on the age of the pup, duration of the separation procedure, gender and rodent strain of investigation [29, 116, 138, 190, 191]. These differential effects are related to the development of the brain, which shows profound changes during the first two weeks of life [169]. Additionally, maternal behaviour plays a very important role in the pup's development. In fact, it is the quality of maternal behaviour encountered by the pup upon reunion that plays a significant role in determining individual differences in stress-responsiveness of the progeny [43].

1.5 Scope and outline of the thesis

1.5.1 Rationale and objectives

As described in the various sections above, adverse early life events can program the susceptibility of the stress system for stressful experiences later in life. Early life adversity therefore may form a risk factor in subjects who already have a genetic predisposition to develop stress-related psychiatric disorders. The separation of infants from their mother is used as a laboratory model to study the immediate and long-term consequences of early adversity. For this purpose in rodents the most frequently used paradigms are daily handling, repeated maternal separations for 3-6 hours and a single 24 hours maternal deprivation. The outcome of each of these treatments is different depending on age, gender and strain of the pup and also on the duration and frequency of the separation.

With respect to the outcome of daily handling and repeated maternal separations most data refer to long-term effects (***section 1.4.4***) and only a limited database is available on the immediate effects of these procedures (***section 1.4.3***). In contrast, in numerous studies only the short-term effects have been examined of a single 24 hours maternal deprivation (***section 1.4.5***) and long-term effects are sparse, especially in mice (***section 1.4.6***). The studies described in this thesis were done to fill these gaps and are presented in two parts. The objective of the studies in the first part of this thesis ("Repeated maternal separations"; ***Chapters 2*** and ***3***), is to determine the short-term cumulative effects of repeated maternal separations on the HPA axis (re)activity. In the second part ("Maternal deprivation"; ***Chapters 4*** to ***6***) the aim is to determine the long-term effects of a single 24 hours maternal deprivation.

1.5.2 Hypotheses and approaches

Part I "Repeated maternal separations": The data described in ***sections 1.4.3*** and ***1.4.4*** have led to the hypothesis that repeated maternal separations produce a sustained activation of the HPA axis. Hence, excessive circulating levels of corticosterone are expected. For this purpose an 8 hours maternal separation procedure was applied that was repeated for three consecutive days. CD1 mice were used in the experiments and the separation procedures were applied between postnatal days 3-5. As read out parameters of the neonatal HPA axis activity circulating ACTH and corticosterone levels were measured. The levels of glucose and ghrelin were measured as indices of the effect of food deprivation associated with the absence of maternal care and feeding. In addition, mRNA expression was measured of markers for neuronal activation (*c-fos*), HPA axis activity (POMC, CRH, MR, GR) and metabolism (NPY). The effects exerted by maternal separation were measured under basal conditions and after exposure to novel environment. Moreover, the response to separation and novelty was also measured under conditions of selective blockade of the either one of the two corticosteroid receptor types. For this purpose MR and GR antagonists were administered either at the beginning of the separation period or at the start of the novelty procedure. The results may shed light on the role of corticosteroid feedback control in early life HPA activity under the various maternal care conditions.

Part II "Maternal deprivation": Long-term effects of a single 24 hours maternal deprivation on cognition and endocrine responsiveness have been observed in rats (see ***section 1.4.6***). In this second part the hypothesis is tested that this paradigm produces a similar outcome in mice and that it is therefore preceded by an altered development trajectory of the HPA axis. To determine these effects the consequences of a single 24 hours maternal deprivation were measured on cognitive performance and endocrine responsiveness in CD1 mice at 6 months of age. A single 24 hours maternal deprivation was also applied at different ages during the SHRP to determine the effects on HPA axis reactivity at weaning. Finally, the effect of maternal deprivation early in the SHRP was investigated on the further development of the HPA axis.

1.5.3 Outline of this thesis

In mice repeated maternal separations have fairly consistent and robust long-term effects on both the cognitive performance and the endocrine stress response, at least in a subgroup of animals (reviewed in [104, 154, 164]). Literature on short-term consequences measured in this model on HPA axis (re)activity during the SHRP is sparse. To better understand and appreciate the long-term consequences using this paradigm, the cumulative immediate effects of repeated maternal separations in CD1 mice were measured. First, the effects of repeated 8 hours maternal separations at postnatal days 3, 4 and 5 were determined on (1) the HPA axis responses to maternal separation in the absence or presence of an additional novelty stressor and (2) the mRNA expressions of selected HPA axis and stress markers in brain and pituitary (***Chapter 2***). Both groups were compared with results observed for experimentally-undisturbed controls and

with the outcome obtained by a single 24 hours maternal deprivation (as described in ***Chapter 7***). *C-Fos* and CRH mRNA expression were measured in the PVN in addition to plasma ghrelin levels and NPY mRNA expression in the arcuate nucleus of the hypothalamus as markers for the metabolic pathway known to be activated by prolonged maternal absence [171]. In the subsequent chapter (***Chapter 3***) the effects of glucocorticoid feedback were explored as a possible explanation for the immediate effects observed in ***Chapter 2***. The HPA axis feedback via the MR and GR was examined using selective antagonists administered in such a way that dissociation between their involvement in response to maternal absence and to novelty became possible.

In contrast to the well documented long-term effects of repeated maternal separations, the single 24 hours maternal deprivation model is best known for its immediate effects on the HPA axis (reviewed in [39, 114, 149, 150, 195]). In rats, but not in mice, only in a few studies long-term effects have been reported [39, 109, 110, 138]. Therefore, it was first investigated if a single 24 hours maternal deprivation in CD1 mice can affect cognitive performance in a spatial learning task and the endocrine response to a stressful event later in life (***Chapter 4***). To gain more insight in the pattern of effects observed in adult mice, experiments were designed (1) to compare the effects at weaning of a single 24 hours maternal deprivation applied at different ages during the SHRP on endocrine responsiveness and on basal mRNA expression of selected HPA axis markers (***Chapter 5***) and (2) to study the consequences of a single 24 hours maternal deprivation at postnatal day 3 on the further development of the HPA axis and the duration of the SHRP (***Chapter 6***).

In ***Chapter 7*** the results of the studies described in ***Chapters 2*** to ***6*** will be summarised and discussed in a broader perspective and conclusions are drawn. Finally, it will be shown in ***Appendix I*** that in male and female CD1 mice the degree of novelty is reflected by the corticosterone response to a stressor but at the same time contains a methodological consideration for the application of tail sampling in mice.

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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).

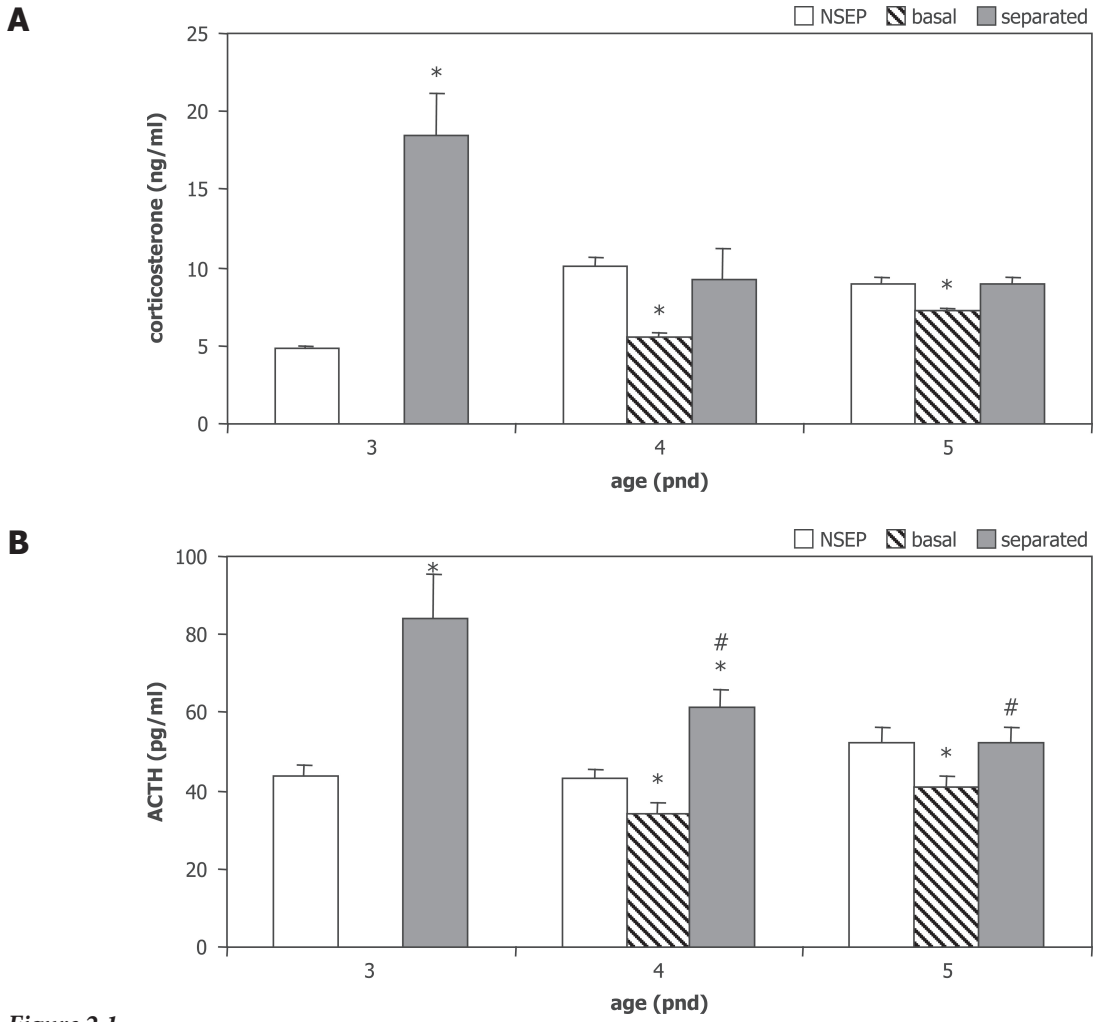

Figure 2.1
Non-separated (NSEP), basal and maternal separation-induced plasma corticosterone (***A***) and ACTH (***B***) levels in mouse pups tested at different ages (non-separated at 9:00 hours; basal: basal levels at 9:00 hours of pups maternally separated for 8 hours on the preceding day(s); separated: 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

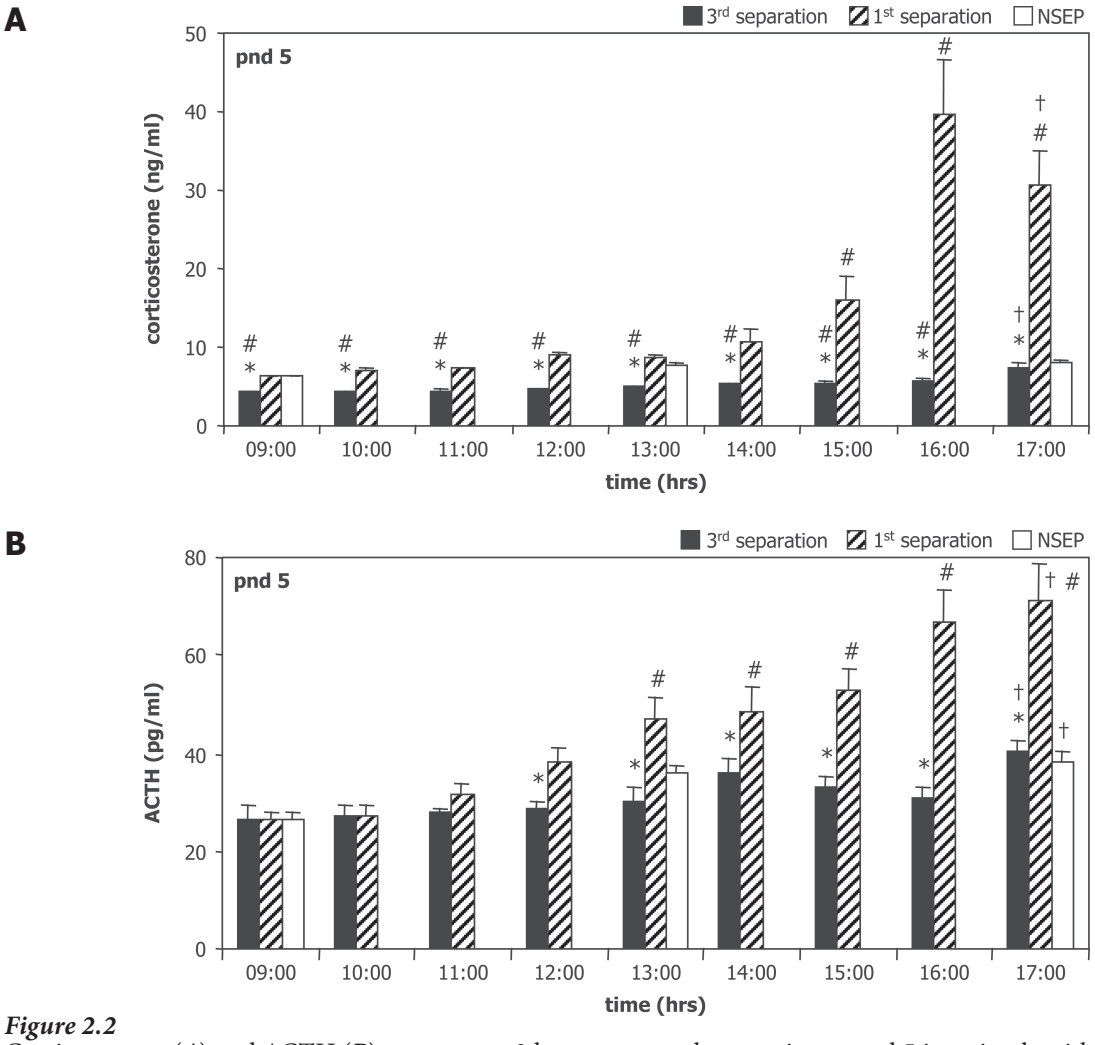

Figure 2.2
Corticosterone (***A***) and ACTH (***B***) response to 8 hours maternal separation at pnd 5 in animals with no previous history of separation (1st separation) and in animals previously exposed to 8 hours separation at pnds 3 and 4 (3rd separation). Non-separated animals (NSEP) were left undisturbed 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, $P<0.05$).

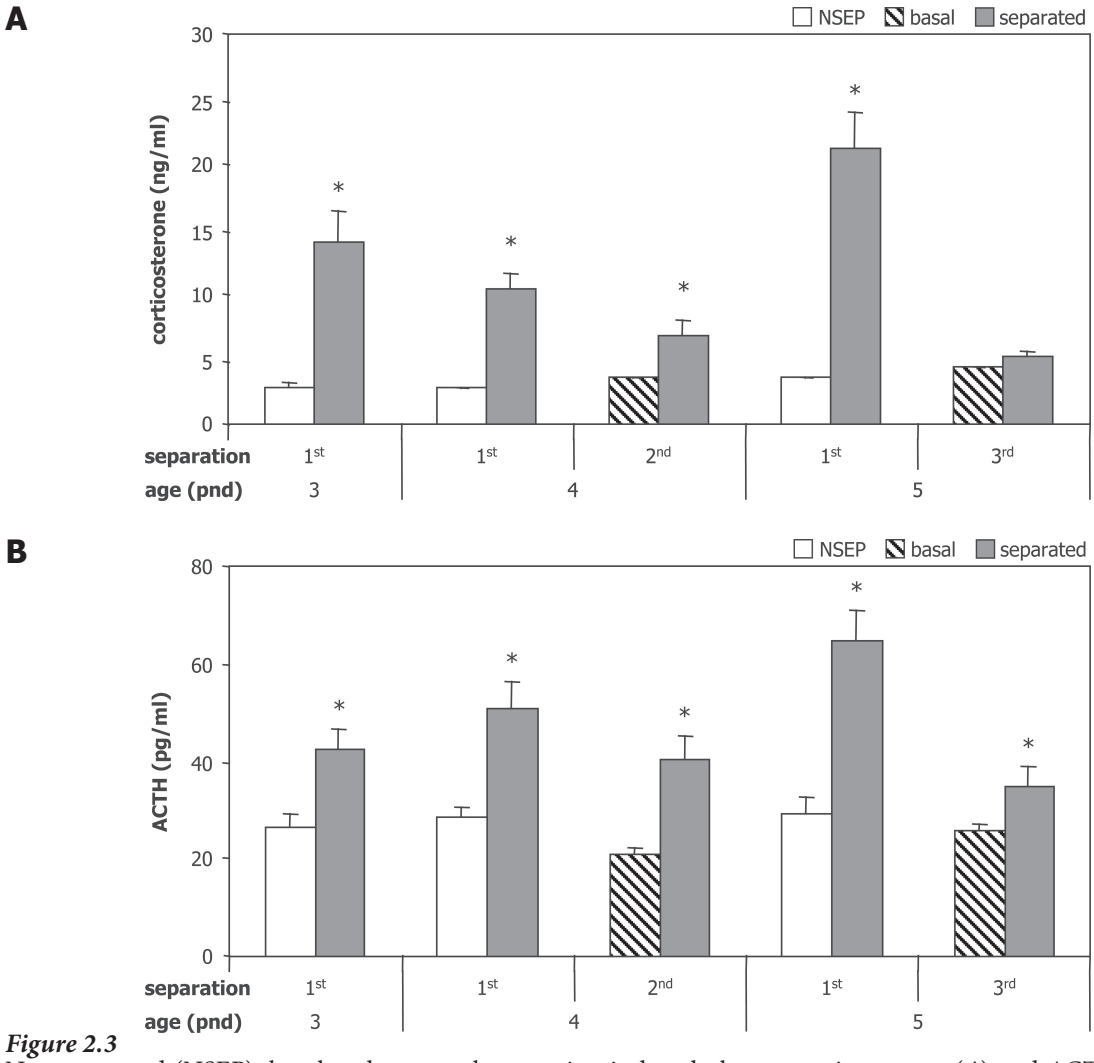

Figure 2.3
Non-separated (NSEP), basal and maternal separation-induced plasma corticosterone (***A***) and ACTH (***B***) levels in mouse pups tested at different ages ("1st": first 8 hours of maternal separation at pnds 3, 4 or 5; "2nd": separated at pnds 3 and 4; "3rd": separated at pnds 3, 4 and 5; 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).

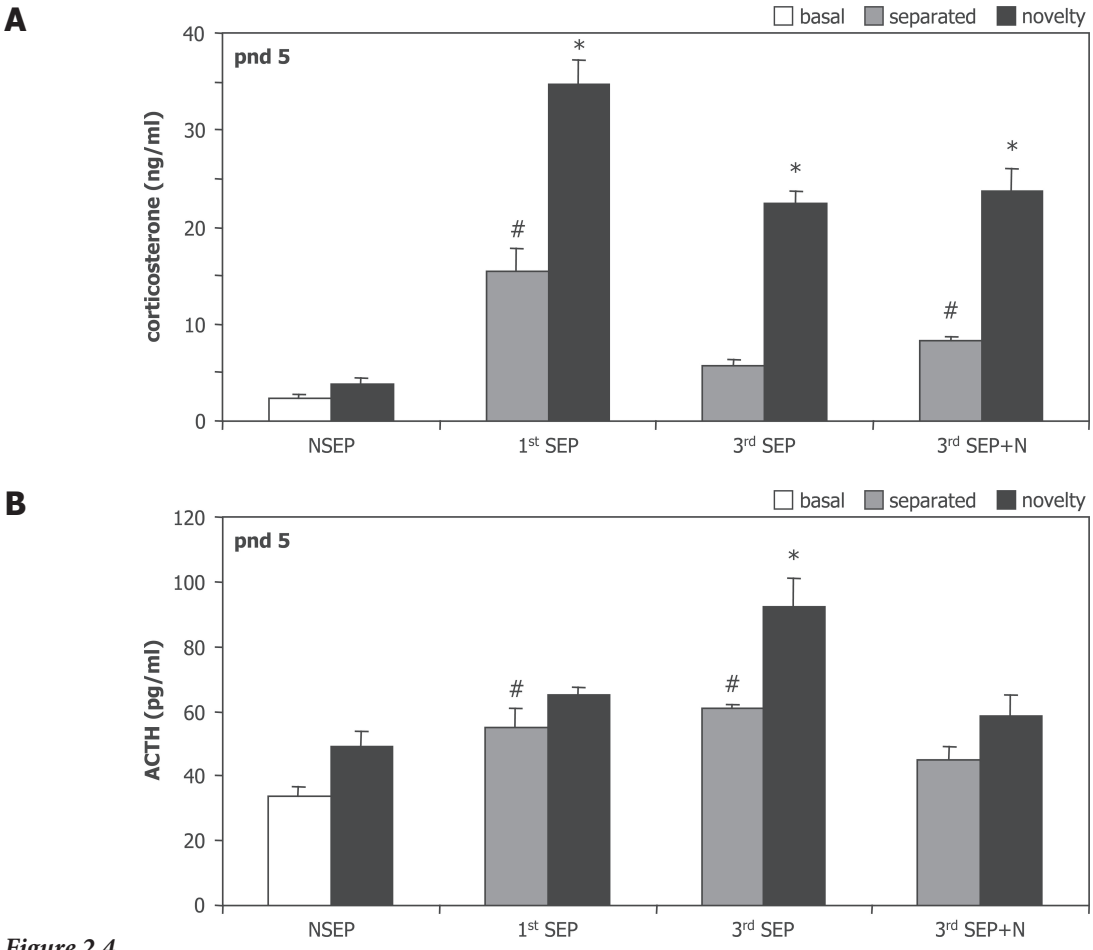

Figure 2.4
Basal, separation and novelty (30 minutes isolated exposure to a new environment) induced plasma corticosterone (***A***) and ACTH (***B***) levels at pnd 5. NSEP and 1st SEP had no previous history of treatments. 3rd SEP animals were exposed to 8 hours separation at pnds 3 and 4. 3rd SEP+N 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.

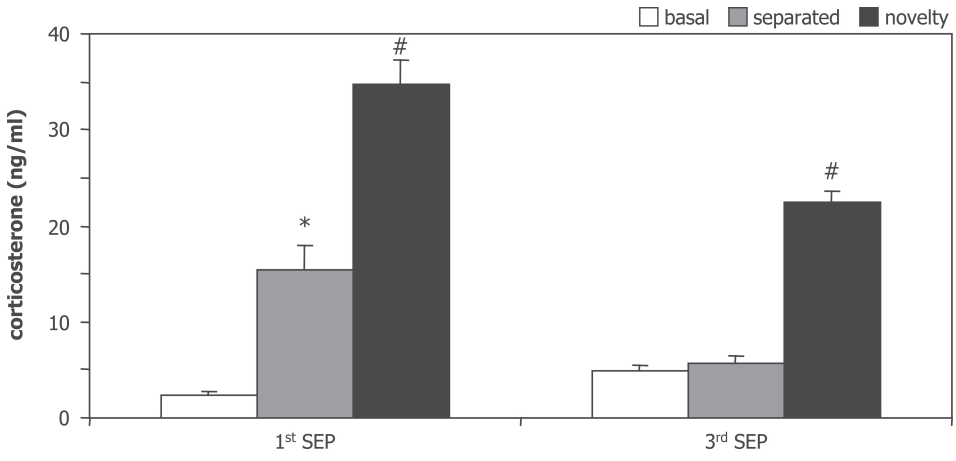

Figure 2.5
Corticosterone in animals at pnd 5 with no previous history of separation (1st SEP) and in animals previously exposed to 8 hours separations at pnds 3 and 4 (3rd SEP). Corticosterone response was measured after 8 hours of maternal separation and after 8 hours of maternal separation with an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

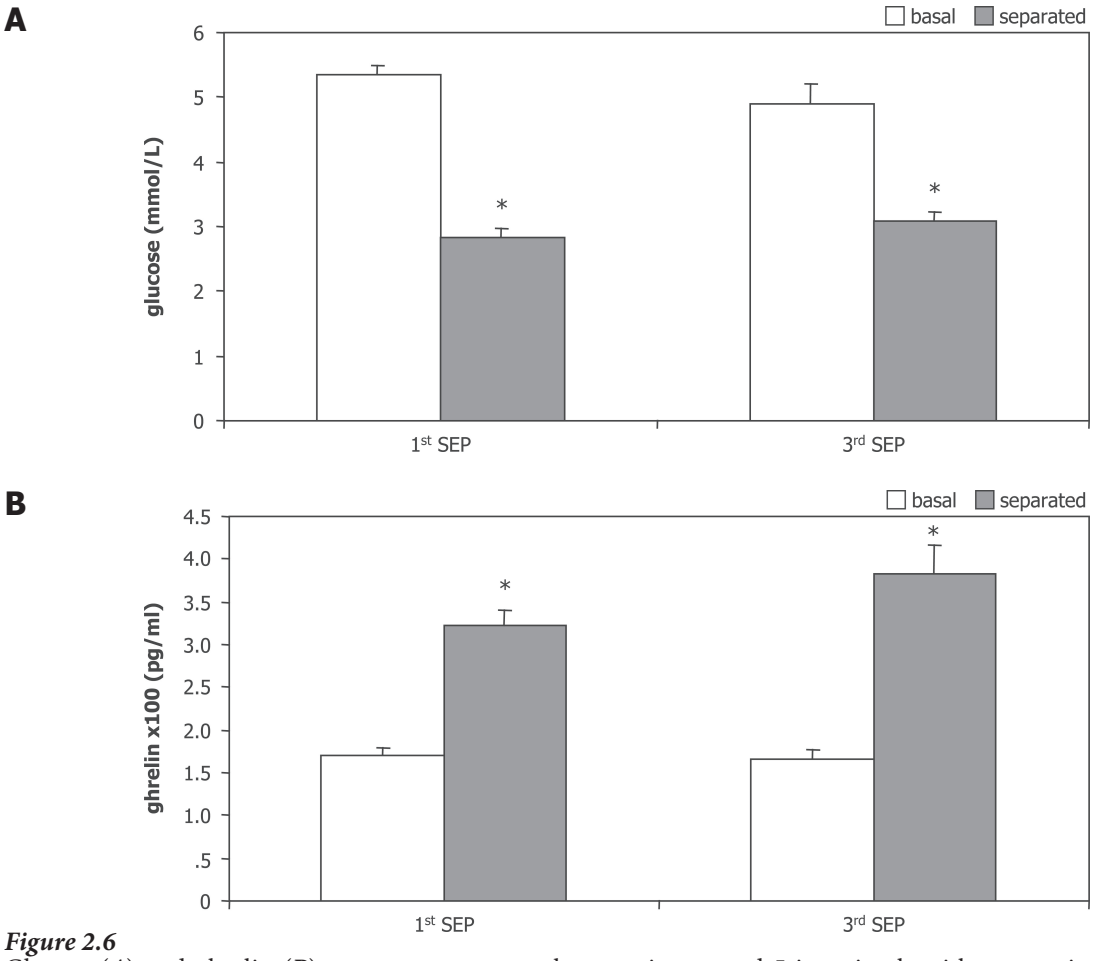

Figure 2.6
Glucose (***A***) and ghrelin (***B***) response to maternal separation at pnd 5 in animals with no previous history of separations (1st SEP) and in animals previously exposed to 8 hours separation at pnds 3 and 4 (3rd SEP). Data represent mean ± S.E.M. * $P<0.05$ (significant from basal).

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.

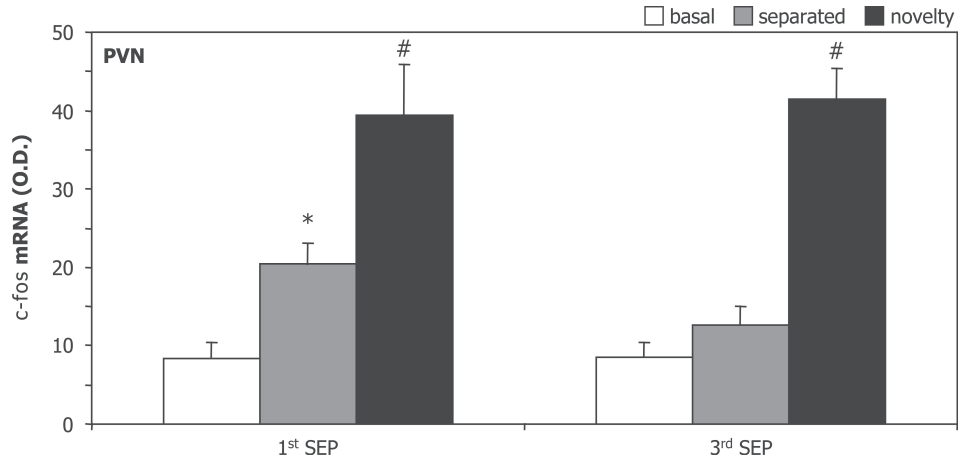

Figure 2.7
C-fos mRNA expression measured in the PVN at pnd 5 in animals with no previous history of separation (1st SEP) and in animals previously exposed to 8 hours separations at pnds 3 and 4 (3rd SEP). Expression was measured after 8 hours of maternal separation and after 8 hours of maternal separation 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.

2.6 Acknowledgements

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The role of brain corticosteroid receptors in HPA axis adaptation to repeated maternal separations of newborn mice

Chapter 3

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3.1 Abstract

In previous studies we observed that the CD1 mouse infant's hypothalamic-pituitary-adrenal (HPA) axis readily desensitises to repeated daily 8 hours separations from the dam, but remains responsive to a novelty stressor. The objective of the current study was to examine if this neuroendocrine desensitisation to maternal absence was due to enhanced glucocorticoid feedback. For this purpose the effect of a mineralocorticoid (MR) or glucocorticoid (GR) receptor antagonist was measured on circulating corticosterone levels.

We obtained the following results: (1) The GR antagonist mifepristone amplified at postnatal day (pnd) 5 the infant's corticosterone response to the first maternal separation, but became ineffective if the pups were exposed the preceding days to repeated maternal absence, the procedure that induced desensitisation of the HPA axis. (2) The GR antagonist caused generally a small decrease rather than an increase of basal circulating corticosterone levels during the stress hypo-responsive period at pnds 5 and 8. However, upon a subtle HPA activation achieved by a mild immune challenge, the GR antagonist became active in interfering with glucocorticoid feedback and triggered a profound corticosterone increase. (3) Blockade of the MR by spironolactone resulted in small, but significant increases in circulating corticosterone levels under basal conditions 8 and 24 hours after injection. This MR antagonist-induced increase in corticosterone was also observed after the third separation, but not if animals were separated for the first time. Then, corticosterone actually decreased. (4) During novelty a brief exposure to the MR antagonist enhanced the corticosterone response in the single and repeatedly separated pups, while GR antagonism under those short term conditions was not effective.

In conclusion, the findings exclude an enhanced glucocorticoid feedback as a mechanism underlying the desensitisation of the HPA axis in response to repeated maternal separations and support the role of the GR in maintenance of the stress hypo-responsive period (SHRP). The results also indicate operation of an MR-responsive mechanism during the SHRP that mostly restrains HPA activity under basal, stressful and maternal separation conditions.

3.2 Introduction

Mice have a stress hypo-responsive period (SHRP from postnatal day (pnd) 1-12 [30]), which is characterised by stable low circulating levels of corticosterone. This implies that mild stressors, which trigger a profound ACTH and corticosterone response in adults, do so only weakly in the newborn animal. However, certain stimuli as interleukin-1, an important mediator of the inflammatory response to infection, are able to elicit an ACTH and corticosterone response during the SHRP [9, 11]. Upon separation of the pups from their mother the HPA axis emerges from the SHRP, indicating that the hypo-responsiveness is (partially) dependent on the presence of the mother [5, 12]. In response to this separation, basal levels of ACTH and corticosterone are increased and the HPA axis becomes responsive to mild stressors, which now can trigger a burst

of ACTH and corticosterone release [24, 31, 34].

Repeated daily separations of mother and pups during the SHRP is an established model to study the long-term consequences of early adversity, which induces at adulthood increased HPA axis responsiveness and behavioural fearfulness, at least in a subgroup of animals [10, 13, 16, 20, 22, 23, 27, 40]. The mechanism underlying these lasting effects on brain and behaviour is thought to be triggered by presumed daily activations of the HPA axis induced by repeated maternal separations [15]. However, we recently showed that pups readily adapt to the repeated absence of the dam, since the HPA response to maternal absence already desensitises at the second separation and the response is virtually absent after the third separation period. However, the increased responsiveness to novelty stress is maintained and even enhanced after repeated maternal separation, indicating that the reactivity of the stress system has remained on alert. The desensitisation of the HPA axis response to repeated maternal absence and the response to novelty is reflected in *c-fos* mRNA expression pattern in the paraventricular nucleus of the hypothalamus (PVN). In contrast, we excluded changes of peripheral metabolic factors as glucose or ghrelin as mediators of the desensitisation [***Chapter 2***]. These findings suggest that the neuroendocrine desensitisation to repeated maternal absence is of central origin.

Enhanced glucocorticoid inhibition has long been argued as one of the main causes for the stress hypo-responsiveness during early development [28, 29, 41]. The actions of glucocorticoids on basal and stress-induced HPA regulation are mediated by mineralocorticoid and glucocorticoid receptors (MR and GR, respectively). These receptors are already expressed in the neonatal brain, although their ontogenetic patterns are different. MR mRNA in limbic brain is already relatively abundant at birth and GR mRNA is still rather low [30], but already expressed in the stress regulatory centers [25, 37]. Schmidt *et al.* [32] recently showed that treating 8 days old control mice with an MR antagonist slightly enhanced circulating corticosterone levels. By blocking the GR a profound response of ACTH and corticosterone release was observed. The latter finding proved the significance of glucocorticoid feedback mediated by the GR in maintaining stress hypo-responsiveness during the SHRP [7, 28, 32, 41].

In the current studies the hypothesis was tested that the disappearance of the HPA response to repeated maternal absence during the SRHP is due to an enhanced glucocorticoid feedback. For this purpose MR and GR antagonists were administered to assess the role of corticosterone feedback signals in the maintenance of the low HPA axis activity after the third separation and during exposure to novelty. The results demonstrate that GR is not involved in the desensitisation of the HPA axis upon repeated maternal separation and that central MR-mediated inputs to the PVN remain responsive under these conditions.

3.3 Materials and Methods

3.3.1 Animals

In this study 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. These 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).

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).

3.3.2 Procedures of separation and novelty exposure

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 was placed in an 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 deprivation period or until testing. Non-separated litters were left undisturbed. Novelty exposure occurred by placing the pups individually in a clean novel cage on a heating pad for 30 minutes.

3.3.3 Antagonist administration

Pups were injected subcutaneously, according to the 'experimental designs', with either vehicle, GR antagonist (mifepristone, 100 µg/g body weight) or MR antagonist (spironolactone, 50 µg/g body weight) using either NaCl with 0.4% Tween80 or polyethylene glycol (PEG) as a solvent. Although both antagonists dissolve well in PEG, we chose NaCl with 0.4% Tween80 as an alternative solvent to avoid a too large injection volume in our 5 days old pups (Experiments I – III) as compared to the 8 days old pups in a study previously performed in our lab [32]. Injection volumes for NaCl with 0.4% Tween80 were always adjusted to 2 µl/g body weight, whereas the volumes for PEG were adjusted to 6 µl/g body weight. However, to exclude a possible effect on the activity of the HPA axis we performed an additional study (Experiment IV) comparing both solvents.

3.3.4 Experimental designs

Experiment I: The objective was to investigate the involvement of glucocorticoid feedback mediated by MR or GR in the desensitised response to maternal separation using antagonists for these receptors. Mice were subjected to four different conditions: Mice were either separated for the first time (1st SEP) or separated for the third time (3rd SEP). Two control groups were included to measure the response to injection itself, without or with previous experience to maternal

separation at pnds 3 and 4 (no SEP and 3rd CON, respectively) . On pnd 5, all animals were injected either with vehicle (NaCl with 0.4% Tween80), the MR antagonist or the GR antagonist and marked with Fuchsine staining (100 mg Fuchsine dissolved in 100 ml MiliQ H_2O with 4% phenol and 10% ethanol). Animals were sacrificed 8 hours after injection (17:00 hours). Fixed factors were TREATMENT (no SEP, 1st SEP, 3rd CON and 3rd SEP) and INJECTION (vehicle, MR and GR antagonist).

Experiment II: To determine whether the MR or GR are involved in the apparent sensitised response to novelty after repeated maternal separations, pups were treated with a selective antagonist for these receptors. At pnd 5, one male and one female in each treatment group were not subjected to novelty stress to provide a reference value (control: either 'basal' or 'separated' for 8 hours of maternal care). The remaining pups were injected after 8 hours of maternal separation with vehicle, MR or GR antagonist (dissolved in NaCl with 0.4% Tween80) and immediately thereafter subjected to 30 minutes novelty stress. Pups from the "no SEP" group were directly placed in the novelty cage, whereas the "1st SEP" and "3rd SEP" were subjected to 8 hours maternal absence before placement in the novelty cage. The fixed factors were TREATMENT (no SEP, 1st SEP and 3rd SEP) and INJECTION (no injection (basal), vehicle, MR and GR antagonist).

In Experiment III the effect of the duration of exposure and the age during the SHRP (early or late) on HPA axis activity to either an MR or GR antagonist was investigated. Pups 5 or 8 days of age were injected subcutaneously with vehicle, MR or GR antagonist (dissolved in NaCl with 0.4% Tween80) for 8 or 24 hours. The first injection was administered at 9:00 hours. Animals selected for the 8 hours time point were decapitated the same day at 17:00 hours. Animals selected for the 24 hours time point were injected twice more at 17:00 and 01:00 hours to maintain a blocked MR or GR and were sacrificed the next morning at 9:00 hours. The fixed factors were AGE (pnd 3 and 8), DURATION (8 and 24 hours) and INJECTION (vehicle, MR and GR antagonist).

In Experiment IV the effect of the solvent on HPA axis activity was investigated. Pups 5 days of age were either sacrificed immediately or injected with vehicle or GR antagonist using either NaCl with 0.4% Tween80 or PEG and then sacrificed 8 hours later. The fixed factors were SOLVENT (NaCl with 0.4% Tween80 and PEG) and INJECTION (no injection, vehicle and GR antagonist).

3.3.5 Collection of blood plasma

At the specific time points described in Experiments I to IV, 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. Plasma samples were stored frozen at -20°C until determination of corticosterone and cytokine concentrations.

3.3.6 Hormone and cytokine analyses

Per experiment plasma corticosterone concentrations were measured separately using a

commercially available radio immunoassay (RIA) kit containing ^{125}Iodine labelled corticosterone (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.

For Experiment IV, plasma C-reactive protein (CRP) concentrations were measured using a using a commercially available ELISA (Life Diagnostics Inc., USA). At the Luminex Core Facility (University Medical Center, Dept. of Pediatrics, Utrecht, the Netherlands) plasma concentrations of several cytokines were determined. The Bio-Plex system employing the Luminex multi-analyte profiling technology (xMAP), allows individual and multiplex analysis of up to a hundred different mediators in a single well containing a sample volume of 50 µl [4].

3.3.7 Statistical analysis

In each experiment every individual group contained animals from at least three different litters (*n*=12). Data of each experiment were analysed by analysis of variance (ANOVA) with their respective main factors and the level of significance set at $P<0.05$. When appropriate this was followed by Tukey's *post hoc* comparisons. The initial analyses included sex as a factor, but once it was determined that sex was not a significant factor, data were collapsed across this variable. All data are presented as mean ± S.E.M.

3.4 Results

3.4.1 <u>Experiment I</u>: To investigate the involvement of glucocorticoid feedback mediated by MR or GR in the desensitised response to maternal separation using antagonists for these receptors.

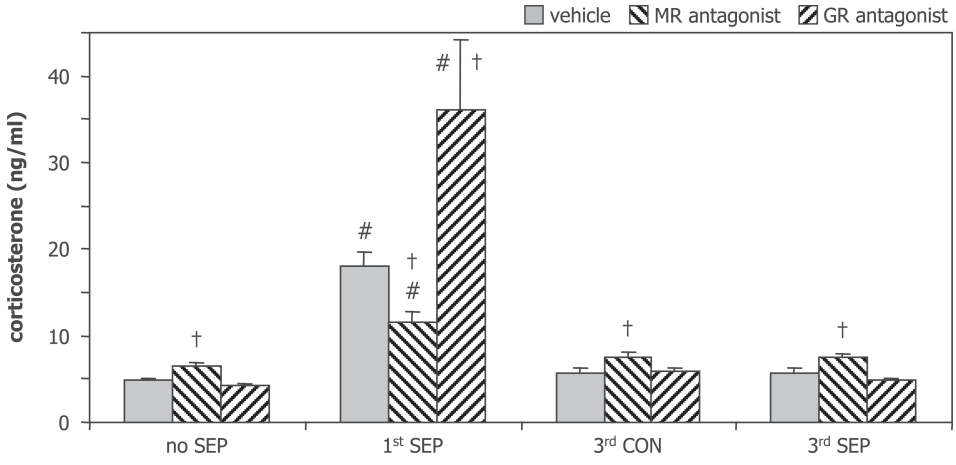

Figure 3.1
Corticosterone response to maternal separation in the presence of either an MR (spironolactone) or GR antagonist (mifepristone) in animals separated from their mother for 8 hours (1st SEP and 3rd SEP), or left with their mother (no SEP and 3rd CON). The 1st SEP group are animals with no previous history of separation, whereas the 3rd SEP group are animals previously exposed to 8 hours separations at pnds 3 and 4. No SEP, which served as a control for the 1st SEP group, was left undisturbed until testing. 3rd CON, which served as a control for the 3rd SEP group, was previously deprived for 8 hours of maternal care at pnds 3 and 4. Injections were given at the start of the 8 hours separation period. Data represent mean ± S.E.M. † $P<0.05$ versus vehicle (within treatment group), $^{\#}$ $P<0.05$ versus no SEP.

3.4.1.1 Corticosterone (***Figure 3.1***)

A main effect was observed of treatment (F(3,116)25.57, *P*<0.001) and injection (F(2,116)3.48, *P*<0.05) as well as an interaction between treatment and injection (F(6,116)6.50, *P*<0.001), indicating that the effect of injection depends on the treatment group. Vehicle-treated pups experiencing maternal separation for the first time (1st SEP) showed increased corticosterone levels compared to their controls (no SEP), whereas a third separation did not affect corticosterone levels anymore (3rd SEP versus 3rd CON). Blocking the MR slightly, but significantly increased corticosterone levels in the no SEP, 3rd CON and 3rd SEP groups, while it suppressed corticosterone secretion in the 1st SEP group. Blocking the GR only affected corticosterone levels in the 1st SEP group, resulting in increased secretion. After three times maternal separation MR, but not GR antagonism could affect corticosterone secretion.

3.4.2 Experiment II: The objective was to determine if an altered MR or GR feedback is involved in the sensitised response to novelty after repeated maternal separations by using selective antagonists for these receptors.

3.4.2.1 Corticosterone (***Figure 3.2***)

A main effect was observed of treatment (F(2,133)88.62, *P*<0.001) and injection (F(3,133)38.30, *P*<0.001). *Post hoc* analyses revealed that in control mice corticosterone secretion increased in response to a first maternal separation (no SEP, basal versus 1st SEP, separated; *P*<0.001), but not anymore in response to a third separation (no SEP, basal versus 3rd SEP, separated). However, in all treatment groups, injection plus exposure to a novel environment increased circulating

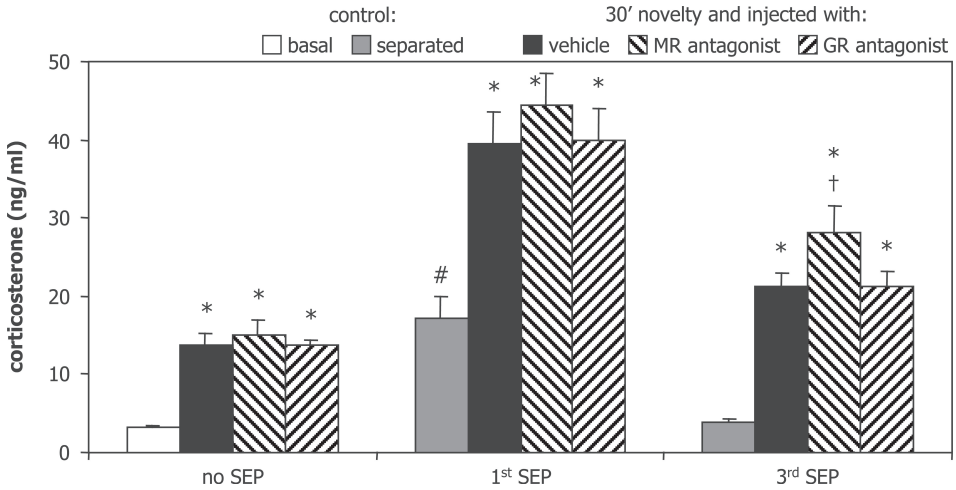

Figure 3.2
Corticosterone response to novelty in the presence of either an MR (spironolactone) or GR antagonist (mifepristone) in animals separated from their mother for 8 hours (1st SEP and 3rd SEP), or left with their mother (no SEP). The 1st SEP group are animals with no previous history of separation, whereas the 3rd SEP group are animals previously exposed to 8 hours separations at pnds 3 and 4. No SEP served as a control for the 1st SEP group and was left undisturbed until testing. Injections were given at the end of the 8 hours separation period right before the 30 minutes novelty exposure. Data represent mean ± S.E.M. * *P*<0.05 versus basal (no SEP) or versus starting levels after 8 hours of maternal separation (1st SEP and 3rd SEP), # P<0.05 versus basal (no SEP), † *P*<0.05 versus vehicle (within treatment group).

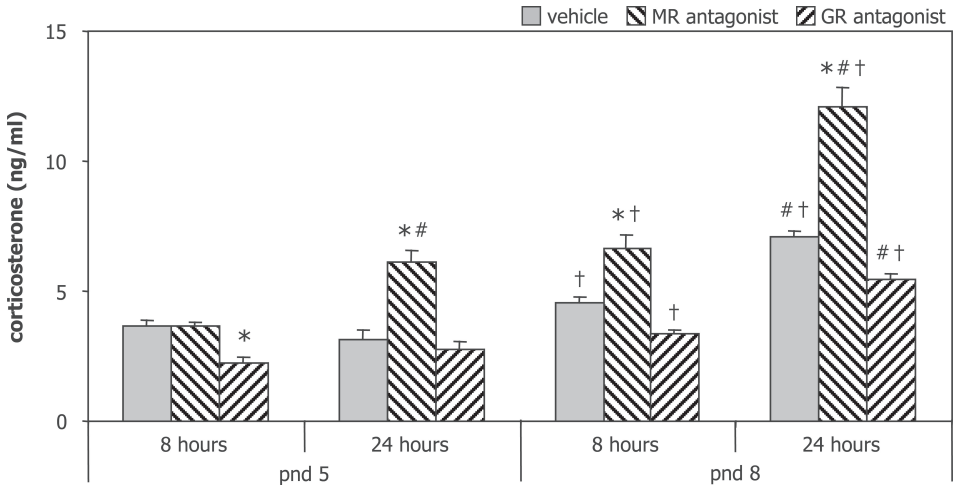

Figure 3.3
Corticosterone concentrations in 5 and 8 days old mice. Mice were treated with either MR (spironolactone) or GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 (vehicle). Data represent mean ± S.E.M., * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

corticosterone levels when compared to control levels ($P<0.001$). Blocking the MR affected corticosterone levels in the 3rd SEP group, resulting in higher corticosterone levels compared to vehicle-treated pups ($P<0.05$). Blocking the GR did not affect corticosterone levels in any of the treatment groups.

3.4.3 <u>Experiment III</u>: The objective was to test the influence of the age of the animal and the duration of antagonist exposure on the amplitude of the pituitary-adrenal response to injection.

3.4.3.1 Corticosterone (***Figure 3.3***)

We observed, besides a main effect of injection ($F(2,150)107.66$, $P<0.001$), that both the age of the pups ($F(1,150)176.51$, $P<0.001$) and duration of treatment ($F(1,150)88.93$, $P<0.001$) significantly

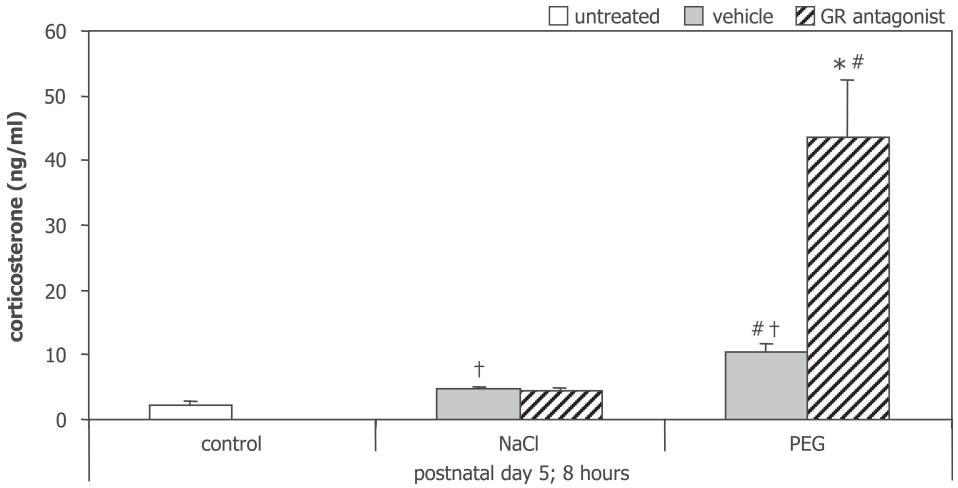

Figure 3.4
Corticosterone concntrations in 5 days old mice after 8 hours treatment with GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 or in PEG. Untreated control animals were naïve to any treatment. Data represent mean ± S.E.M. * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

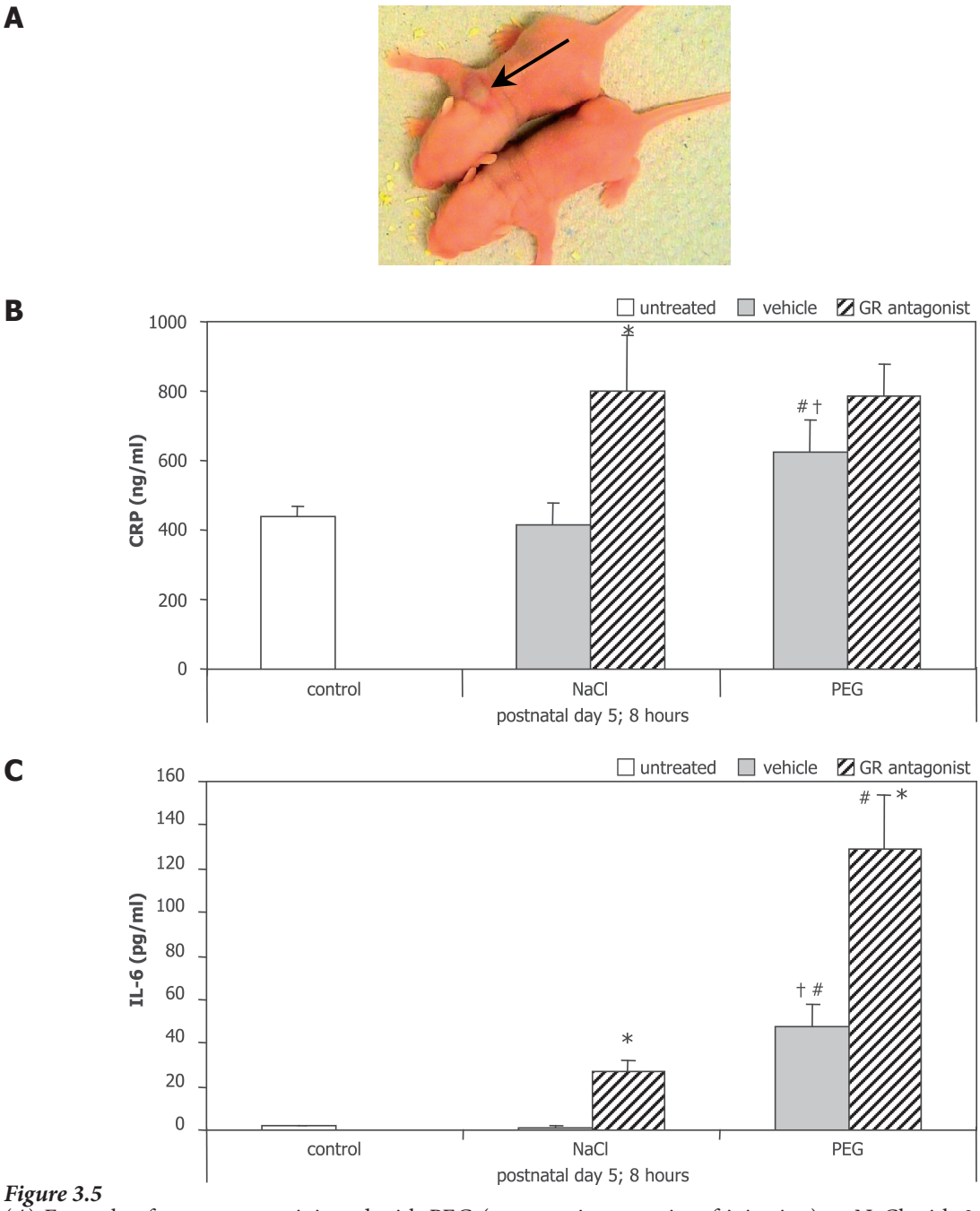

Figure 3.5
(***A***) Example of a mouse pup injected with PEG (arrow points out site of injection) or NaCl with 0.4% Tween80 as vehicle for antagonist delivery. CRP (***B***) and IL-6 (***C***) concntrations after 8 hours treatment with GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 or in PEG. These measurements were performed in the same blood samples as for the corticosterone concentrations of ***Figure 3.4***. Untreated control animals were naïve to any treatment. Data represent mean ± S.E.M. * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

affected corticosterone levels. At pnd 8 the vehicle injection at the 8 hours time point resulted in corticosterone levels that were higher compared the same time point at pnd 5 ($P<0.5$). Furthermore, these levels further increased at the 24 hours time point, whereas this was not observed at pnd 5. Except for the 8 hours time point at pnd 5, the MR antagonist slightly increased circulating corticosterone plasma levels ($P<0.01$). The GR antagonist slightly reduced circulating corticosterone levels ($P<0.05$). Overall, a more pronounced amplitude of the responses was

observed in 8 days old pups as compared to the 5 days old pups.

3.4.4 Experiment IV: The objective was to determine whether the immune challenge of the HPA axis triggered by the injection with PEG as vehicle might interfere with the effect of the MR and GR antagonists.

3.4.4.1 Corticosterone (***Figure 3.4***)

When analysing the corticosterone data we observed both an injection effect (F(1,45)12.01, $P<0.001$) as well as a solvent effect (F(1,45)21.95, $P<0.001$) and an interaction between injection and solvent (F(1,45)12.62, $P<0.01$). When injecting pups with PEG the basal levels of corticosterone after 8 hours increased and this increase was even further enhanced when the GR antagonist was administered ($P<0.001$). However, injecting pups with NaCl with 0.4% Tween80 only slightly raised basal circulating corticosterone levels ($P<0.01$), but this rise was much smaller than when injected with PEG ($P<0.001$). Furthermore, adding the GR antagonist did not further affect these levels.

3.4.4.2 CRP and cytokines (***Figure 3.5***)

Since a reddish circle appeared at the site of injection in the pups that were injected with PEG solvent (***A***) we measured C-reactive protein (CRP) and cytokine levels.

For CRP (***B***) we observed a main effect of solvent (F(1,48)7.54; $P<0.01$), but not of injection. CPR levels in pups injected with PEG were higher relative to NaCl with 0.4% Tween80 as vehicle ($P<0.05$) and compared to uninjected controls ($P<0.05$). Although there were no significant differences in CPR levels in mice receiving either solvent, CPR levels increased significantly in animal receiving the antagonist dissolved in NaCl with 0.4% Tween80, while CRP levels remained high when the it was dissolved in PEG.

For IL-6 (***C***) we also observed a main effect of solvent (F(1,53)19.26; $P<0.001$), but also of injection (F(1,53)37.28; $P<0.001$) and an interaction between solvent and injection (F(1,53)5.24; $P<0.001$). Injection of animals with NaCl with 0.4% Tween80 did not affect basal IL-6 levels, while injection with PEG significantly increased these levels ($P<0.05$). Administration of GR antagonist significantly increased IL-6 levels when it was dissolved in NaCl with 0.4% Tween80 ($P<0.05$). However, IL-6 levels were even further increased when it was dissolved in PEG ($P<0.05$).

3.5 Discussion

The present study shows that the infant's corticosterone response to 8 hours of maternal separation disappears when the procedure is repeated on three consecutive days from postnatal days 3 to 5. However, at the time the corticosterone response to separation is abolished, an additional exposure to novelty still has a profound effect. This finding reinforces our previous observations showing that the pup's HPA axis readily desensitises to repeated separations, but continues to respond to novelty [***Chapter 2***].

In order to test if enhanced corticosterone feedback could explain the abolishment of the pituitary-adrenal response after repeated separations we have administered MR or GR antagonists to the separated pups. The results show that the GR antagonist mifepristone amplified the infant's corticosterone response after the first 8 hour separation, as previously shown also after a 24 hours separation paradigm [32], but became ineffective at the third separation. This finding excludes enhanced glucocorticoid feedback as a mechanism underlying the desensitisation of the HPA response to maternal absence, if the separations of 8 hours were repeated each day.

Enhanced inhibition of the HPA axis during the SHRP via GR has long been argued as the main cause for the hypo-responsiveness during early development [26, 28, 29, 32, 41]. However, in the current studies blockade of GR with the antagonist dissolved in NaCl containing 0.4% Tween80 did under basal conditions not affect circulating corticosterone levels in the 3 to 5 days old neonates, as it did not 24 hours later. The antagonist even quite consistently slightly reduced circulating corticosterone levels ($P<0.05$) at day 5. At first glance our data do not seem to agree with a previous report from this laboratory, demonstrating that GR-mediated feedback maintains the low and stable corticosterone levels characteristic for the SHRP [32]. We have repeated the studies by Schmidt *et al.* under identical conditions both at postnatal day 5 and 8 and neither after a single or two times administration of the GR antagonist a response was measured 8 hours later.

What could be the reason for this paradox? In retrospect, there is no discrepancy. It is likely that in neonatal mice truly basal circulating levels of corticosterone are insufficient to activate the GR. This situation is reminiscent to that in adult animals, where it is since long known that basal levels do not sufficiently occupy the GR and therefore cannot be blocked by a GR antagonist [21]. Thus, it seems that similar to the adult situation GR antagonism only becomes in operation under stress-induced conditions, as this receptor mediates the effects of corticosterone on normalisation and recovery from stress. Hence the conclusion in previous studies that GR mediated feedback maintained hypo-responsiveness is justified, but what could have been the stimulus in the previous studies that had led to the subtle increase in corticosterone levels sufficient to reveal the role of GR-mediated feedback in the SHRP?

Upon repeating the studies we noticed a reddish circle at the site of injection in the pups that were now injected with PEG solvent as used in the previous studies (***Figure 3.5.A***), indicating that the solvent may have influenced the HPA axis by triggering an immune response [2, 42]. Immune system activation of the HPA axis during the SHRP has already been shown in rats [9, 11]. To confirm this we measured the cytokine profile in blood plasma of these mice in response to NaCl with 0.4% Tween80 and PEG alone, or in combination with an anti-glucocorticoid. We measured CRP, an acute phase protein that is elevated in serum as a result of injury, infection or disease [33] and established as a reliable marker for inflammation [19]. As expected the GR antagonist, foreign to the body, triggered an immune response expressed by increased plasma CRP. Surprisingly, PEG alone also induced an immune response, while the NaCl with 0.4% Tween80 vehicle solution did not. Since there is a close reciprocal interaction between the immune and stress system [1], the

observed GR antagonist-induced corticosterone increase with PEG as a solvent has to be judged in the light of an activated HPA axis.

We also measured other cytokines and among these was IL-6, which showed a profound response to PEG. In these experiments we also reproduced that the anti-glucocorticoid in NaCl with 0.4% Tween80 was ineffective 8 hours after. Indeed, also PEG alone caused a slight corticosterone rise apparently sufficient for the anti-glucocorticoid to interfere and to trigger a profound corticosterone response. IL-6 is known to activate the HPA axis, so it appears that a mild inflammatory response is sufficient to uncover the role of glucocorticoid inhibition in the maintenance of the SHRP.

Blockade of the MR gave different results. The corticosterone rise induced by the first 8 hour separation is attenuated, while the MR antagonist produced a small corticosterone response in the pups subjected to three daily separations. A rise in corticosterone was also observed if the animals are older, at 8 days, or when the separation period is extended to 24 hours. Hence, it seems that an MR- rather than a GR-responsive network is still active during repeated maternal absence. Such an MR-responsive pathway could be implicated in the central mechanism underlying desensitisation as was identified in the previous study using *c-fos* mRNA expression in the PVN as a criterion. Such a central pathway may refer to the recent studies of Moriceau *et al.* [17, 18], who showed a switch from a locus coeruleus-olfactory pathway governing attachment to the mother towards the premature development of an amygdala pathway mediating avoidance from adverse conditions. This switch was facilitated by corticosterone, which we showed to be increased under conditions of novelty exposure and inflammation, particular during maternal absence.

The abolished HPA axis response to repeated maternal absence suggests that the pups may be able to predict the return of the mother and thus the reinstatement of maternal care after 8 hours. This situation is reminiscent to experiments showing that 3x 45 seconds stroking of the anogenital region with a wet brush is sufficient to prevent the single 24 hours maternal deprivation induced *c-fos* activation in the PVN and basal increase in plasma ACTH [35, 38, 39]. Subsequent feeding is able to normalise or prevent the rise in corticosterone values [35, 39]. However, in previous studies [***Chapter 2***] we excluded that an altered food intake pattern was the cause of the dynamic changes in the HPA axis upon repeated separations, since circulating glucose and ghrelin levels were not affected. Furthermore, reunion of mother and pups after a prolonged period of separation causes a bout of increased maternal care [14]. This extra attention by the mother also could be involved in the adaptation to repeated separations.

To investigate the role of MR and GR in the novelty response, pups were injected with an antagonist at the end of the 8 hours maternal separation period before applying novelty stressor. This design allowed testing the role of the recently discovered fast non-genomic membrane-mediated MR- and GR-like actions [8, 36]. The data showed that novelty triggered a response both after the first and the third separation, but only the MR antagonist enhanced the novelty-induced corticosterone secretion. GR blockade did not render an effect in our mice, possibly

because the 30 minute time interval is too short for a GR-mediated mechanism to develop.

Interestingly, MR-mediated disinhibition of HPA axis activity is supported by data in both adult rats and mice [3, 6, 21]. The findings therefore suggest that blocking the MR may relieve a tonic inhibition of the HPA axis [7] or more likely inhibits the non-genomic MR-mediated actions in the hippocampus, which would also imply a reduced excitatory outflow from the hippocampus to the GABA-ergic network surrounding the PVN. The latter mechanism, active for MR rather than GR, could explain the rapid enhancement after MR blockade in the novelty-induced stressor. All together, it seems that the MR-mediated control of basal pulsatile HPA activity and its role in sensitivity to stressors is similar in the infant and the adult. Also the GR-mediated actions, which are absent under truly basal activity, become in operation during exposure to mild daily stressors ensuring maintenance of the SHRP.

In conclusion, enhanced GR-mediated feedback underlying the desensitised corticosterone response to repeated maternal separation is unlikely. The previously reported disinhibitory effect on GR antagonists during the SHRP is a striking example how a subtle change in HPA activity characteristic for the SHRP can be amplified when glucocorticoid feedback is inhibited. Finally, it seems that MR-responsive afferents to the PVN are implicated that manage subtle changes in HPA activity during the SHRP under basal conditions and after repeated separations with or without novelty exposure.

3.6 Acknowledgements

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Effects of maternal deprivation on performance in the water maze and swim stress

Chapter 4

L. Enthoven, E.R. de Kloet & M.S. Oitzl

4.1 Abstract

Rat pups subjected to a single 24 hours maternal deprivation show altered stress responsiveness and cognitive performance in the water maze at adulthood. Here we show in 6 months old male CD1 mice deprived of maternal care for 24 hours at postnatal day 8, an initial impairement in reversal learning: relocating the platform revealed perseverance in search for the former platform location. Spatial learning, long-term memory and swim-induced corticosterone responses were not affected. We conclude that reduced flexibility is a subtle long-lasting behavioural change induced by maternal deprivation.

4.2 Introduction

Traumatic early life events can program the susceptibility of the stress system for stressful experiences later in life and influence both behavioural and endocrine responses [18 - 20, 28, 33]. Therefore, they are considered risk factors for the development of mood disorders [2, 9]. To study the consequences of a disruption of postnatal development in rodents maternal deprivation paradigms are used. Maternal deprivation is the separation of mother and pups for a single period of 24 hours during the stress hypo-responsive period (SHRP). In rats, this paradigm resulted in long-lasting effects on cognition and endocrine responses to stressful stimuli [18 - 20, 28, 33].

For example, maternal deprivation of Brown Norway rats delayed acquisition of a spatial learning task until adulthood (at 3 and 12 months of age) and caused a higher degree of persistent behaviour. With increasing age, group differences in performance were gone at 32 months of age. However, the inter-individual differences in the senescent population increased. While control rats showed a normal Gausian distribution for learning, maternal deprivation resulted in an inverted U-shape distribution with more rats performing either very poor or very good [18].

Cognitive impairment as a consequence of aging is influenced by stress experienced throughout life. Though corticosteroids are essential for cognitive performance [3, 16], excessive corticosterone responses to stress have been associated with impaired cognitive performance in various learning tasks [3, 4, 21, 27]. Maternal deprivation results in both immediately increased basal ACTH and corticosterone [19, 28], but also in an age-dependent attenuated or hyper-responsive hypothalamic-pituitary-adrenal axis (HPA axis) [20, 33]. Thus, also a possibly affected endocrine responsiveness has to be taken into account when investigating the consequences of maternal deprivation on spatial learning.

Genetically manipulated mice are becoming more and more important subjects of research. To understand the effects of genetic manipulations and dissociate them from environmental contingencies, knowledge on normal development and the consequences of early life events are crucial. In mice the immediate short-term consequences of maternal deprivation generally show the same effects as observed for rats (for a comparison see [25]). In contrast, long-term effects of a single 24 hours maternal deprivation on cognition or endocrine responses to stress have to

our knowledge, as of yet, not been investigated in mice. Therefore, we studied the consequences of a single 24 hours maternal deprivation at postnatal day (pnd) 8 on both cognition and corticosterone responsiveness in CD1 mice at adulthood (6 months of age). Taking the overlap in immediate effects of maternal deprivation at pnd 8 in CD1 mice with those observed in rats into account [5, 25, 26, 30, 31] we hypothesised that the long-term consequences will also be comparable. We expected a delayed acquisition of spatial learning in the water maze and a hyper-responsive HPA axis to novelty exposure.

4.3 Materials and Methods

4.3.1 Animals

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

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.

4.3.2 Maternal deprivation and weaning

Mothers nursing litters selected for maternal deprivation were removed from their cage and placed in clean type 3 polycarbonate cages at 9:00 hours on pnd 8. 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. Pups were not fed during the deprivation period. After 24 hours (at 9:00 hours on pnd 9), the mothers were reunited with their pups and left undisturbed. Control litters were left undisturbed. At weaning (pnd 28) the pups were placed in all male and all female groups with littermates from the same nest (n=4 per cage) and left undisturbed until testing.

4.3.3 Water maze schedule and procedure

At 6 months of age mice (n=10 per treatment) were tested in the water maze for their spatial learning abilities.

Handling of the animals: Particular attention was paid to handle the mice gently and quietly. Mice were picked up at the base of their tail and placed in the water maze. When search latencies

exceeded 60 seconds, a metal grid (5 x 20 cm) was used to guide the animals to the platform of the water maze and later to remove them from the platform. Upon presentation of the grid, animals climbed onto it and could easily be transported to their home cage. Any unwanted punishment for finding the platform or chasing the mouse through the pool was thus avoided.

Water maze procedure: A pool (white, diameter 140 cm) was filled with warm water (26 ± 1°C) and made opaque by the addition of chalk. A platform (8 cm in diameter) was situated 1 cm below the water surface, invisible for the animal (spatial condition). One free swim trial with no platform present was run before training. The mouse was placed in the middle of the pool and allowed to swim for 120 seconds. For training trials the pool was divided into 4 quadrants with the platform in the middle of one of the quadrants. For each trial, the mouse was placed in the water, with the head facing the wall, at one of four possible starting positions at an intersection between two quadrants. A maximum of 60 seconds was allowed, during which the mouse had to find the platform and climb onto it and remain there for 10 seconds. If the animal did not find the platform, it was guided there with a grid and allowed to stay for 10 seconds. Animals were tested sequentially with an inter-trial interval of approximately 5 minutes. After each trial, mice were placed individually in type 1 polycarbonate cages containing paper sheets under a red-light heating lamp to dry for approximately 3 minutes. Thereafter, mice were either placed in the water for another trial or back in their home cage.

Training schedule: Three days before spatial training in the water maze started, the pool was filled with approximately 2 cm of warm water (26 ± 1°C) and a large flat object to climb on. This was the mice's first contact with water and each mouse was allowed to move around for 120 seconds (water adaptation trial). Water maze training on day 1 started with a 120 seconds free swim in the absence of the platform (before training). This allowed estimation of the ability of the mice to swim and to determine the pre-training swim pattern, *i.e.* their exploratory strategy, indicative for any preferences for a certain part of the pool. Directly thereafter, spatial training with a submerged platform started. Mice performed 26 trials distributed over eight test days. Within a training day, inter-trial intervals were 5 minutes. After the second trial on day 4 (trial 12) the platform was moved to the opposite quadrant to test reversal learning abilities. Between the last trial on day 5 (trial 17) and the first trial on day 20 (trial 18) a two weeks break was introduced to test long-term memory.

For all training trials we assessed the time needed (seconds), speed (cm/s) and distance swum (cm) to find and climb on the platform. Thereafter, we calculated the mean performance of each mouse per day. Total traveled distance indicated the level of general activity. Behaviour was recorded on videotape and analysed by EthoVision 1.95 (Noldus Information & Technology BV, Wageningen, The Netherlands).

4.3.4 Swim stress, blood sampling and corticosterone measurement

At 6 months of age a separate group of male mice (n=10 per group) was tested for their stress responsiveness to swim stress. One day before the actual swim stress a basal blood sample was

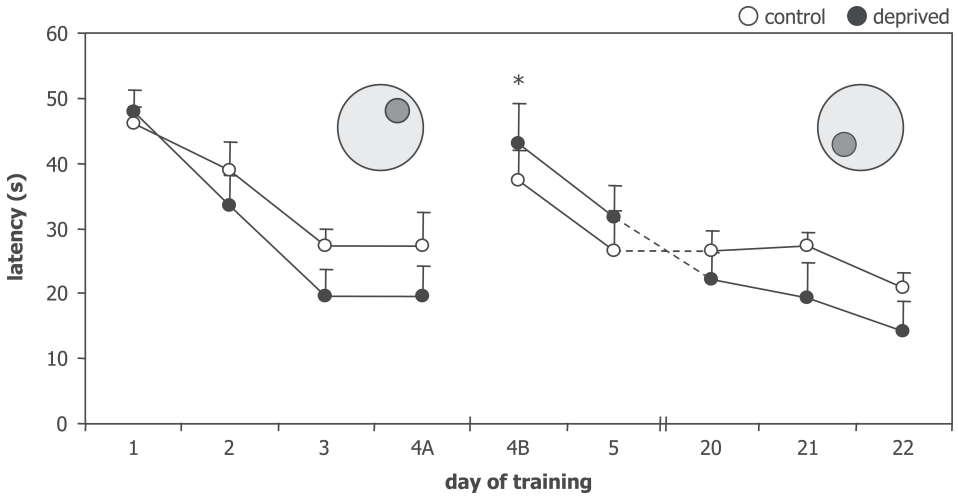

Figure 4.1
Performance of maternally deprived and control CD1 mice in the water maze, presented as the latency (in seconds) to reach the platform. Between 'day 4A' and 'day 4B' the platform position was reversed to the opposite quadrant from the position for 'day 4A'. The change in platform position is illustrated by the inset indicating a water maze with the respective platform positions. The dashed line (---) represents the two weeks delay in between 'day 5' and 'day 20'. Data represent mean ± S.E.M. of daily trials, * $P<0.05$ significant from 'day 4A' within the same group.

collected via tail incision [6, 7]: a small incision at the base of the tail with a razor blade allowed collection of a 50-100 µl blood sample within 90 seconds after opening of the animal's cage. At the day of testing, the mouse was released in the middle of the pool used for water maze testing. After one minute, the mouse was removed from the water using a grid and placed in its home cage under a red-light heating lamp for another three minutes. At 15, 30, 60, 90 and 180 minutes after the mouse was placed in the water maze a blood sample was collected.

Blood plasma was collected individually in potassium-EDTA coated 10 ml tubes (1.6 mg EDTA/ml blood; Sarstedt, Germany). All samples were kept on ice and later centrifuged at 13000 rpm for 15 minutes at 4°C. Blood plasma was transferred to Eppendorf tubes for corticosterone determination and stored at –20°C until further analysis. Corticosterone was measured using a commercially available radio immunoassay (RIA) kit containing ^{125}Iodine labelled corticosterone (MP Biomedicals INC., CA, USA). Vials were counted for 2 minutes in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

4.3.5 Statistical analysis

Performance in the water maze and corticosterone responses to stress were analysed using a general linear model. TREATMENT (control, deprived) was the between subjects factor. The within subjects factor was DAY for water maze performance and TIME (minutes) for the response to swim stress. Comparisons at different time points were tested *post hoc* with independent samples t-tests (between groups), or Wilcoxon paired samples tests (between days). Statistical significance was accepted at $P<0.05$. The performance of the water maze is presented in average performance per day. All data are presented as mean ± S.E.M.

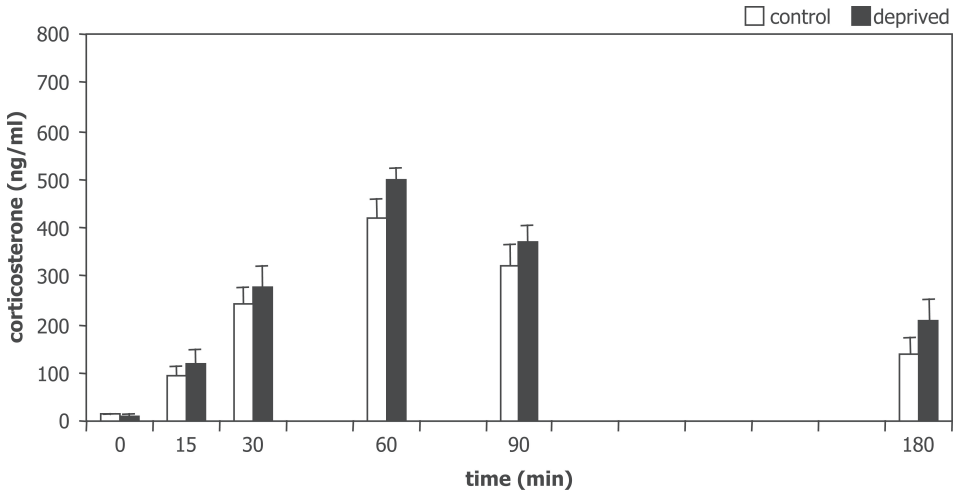

Figure 4.2
Basal and swim stress-induced plasma corticosterone levels (ng/ml) in control and deprived CD1 mice. Data represent mean ± S.E.M.

4.4 Results

4.4.1 Water maze performance (***Figure 4.1***)

In the free swim trial before training, mice of either treatment group showed a randomly distributed exploration pattern of the pool (data not shown). Distances swum were comparable as well (distance (cm): control 1333.76 ± 47.50; deprived 1250.77 ± 67.58). Latency to locate the platform indicated that all mice learned the task (days 1 to 4A; DAY: $F(3,51)17.04$, $P<0.001$), reflected by the decreasing time swum. This performance was not influenced by maternal deprivation. When on the second half of day 4 the platform was moved to the opposite quadrant (*i.e.* day 4B), it took deprived mice significantly longer ($P<0.05$) than controls to find this new position. With further training all mice learned the new position (days 4B to 22; DAY: $F(4,68)11.25$, $P<0.001$). A two weeks break of the training schedule (between days 5 and 20) did not affect the performance.

With increasing numbers of trials mice swum faster (days 1 to 4A; DAY: $F(3,54)6.15$, $P<0.001$). At day 4 mice had reached their maximum speed, which was neither affected by the reversal trial nor by the two weeks break ($P>0.05$; mean velocity (cm/s) at pnd 4B: control 23.18 ± 0.78, deprived 23.04 ± 1.23; at pnd 22: control 23.69 ± 1.31, deprived 21.80 ± 1.43). Though the first four test days the swim speed increased, the decreasing distance traveled indicated that mice were more goal-directed (days 1 to 4A; DAY: $F(3,51)9.84$, $P<0.001$). This was even more prominent during the reversal training (days 4B to 22; DAY: $F(4,68)9.96$, $P<0.001$). When the platform position was switched during the reversal trial deprived mice swum longer distances ($P<0.05$) than control animals ($P=0.44$).

4.4.2 Swim stress (***Figure 4.2***)

The basal corticosterone concentrations were comparable between deprived and control mice. All mice responded similarly to swim stress: with an increase in corticosterone secretion

(TIME: F(5,90)65.25, $P<0.001$). After 3 hours corticosterone had returned to basal levels with no differences between the groups.

4.5 Discussion

CD1 mice readily learned this spatial navigation task, indicated by the decreased latency and distance swam to locate the submerged platform. Maternal deprivation did not influence the acquisition of this task.

Maternally deprived and control mice showed similar learning curves for latency and distance swum. Thus, unlike maternally deprived rats [18], maternally deprived mice did not show a delayed acquisition of the water maze. Interesting is the swim pattern of deprived mice when the platform was relocated opposite to the trained position. In this reversal trial, mice returned persistently to the trained platform position before searching for an escape option elsewhere. They took longer latencies and swam longer distances to find the new platform position, which is in accordance with rat data at 12 months of age [18]. Also in these 6 months old mice we observed a less flexible behavioural pattern. It is the first response to the altered environmental condition, as the learning curve for this new position and the long-term memory established by continuing training after a two weeks break were not affected. We might consider this persistent behaviour adaptive, as long as environmental conditions remain stable.

Corticosteroids facilitate as well as impair cognitive performance [3, 11, 16]. While a context-dependent increase of corticosterone is mainly related to facilitation of learning and memory, long-lasting elevations of corticosterone and periods of stress have rather been associated with impaired cognitive performance [4, 21, 27]. Here, corticosterone responses to swimming were comparably high in maternally deprived and control mice. It is most likely that this task-related increased corticosterone potentiated the consolidation of information and resulted in comparable learning curves and long-term memory in both groups.

Comparing the behavioural and endocrine data of maternally deprived rats and mice, we have to face basic methodological differences between the rat and mouse studies. Factors like species-dependent postnatal period of the SHRP, age of deprivation, age of testing and received maternal care after the deprivation period are likely to affect the outcome. First, independent of the species and postnatal day, the immediate effects of maternal deprivation are very similar. However, long-lasting effects seen in pituitary-adrenal responses to stress, emotionality and cognition that were observed weeks to months later were different. Rats deprived early (pnd 3) or late (pnd 11) during the SHRP showed either a hyper- or hypo-responsive ACTH response to stress at weaning (pnd 20) [29, 30]. In mice, an early (pnd 3) or late (pnd 8) deprivation resulted in a prolonged or unaffected corticosterone response at weaning (pnd 28), respectively [***Chapter 5***]. Avoidance learning in rats was reduced by maternal deprivation at pnd 4, whereas deprivation at pnd 9 (in the middle of the rat SHRP [13, 22, 34]) resulted in enhanced active avoidance and water maze learning [12]. Although the immediate effects of maternal deprivation are comparable

between rats and mice [25], nothing is known about the long-term consequences in mice. One might speculate that, since maternal deprivation at pnd 8 is at the end of the mouse SHRP [23], long-term effects might be less pronounced than expected for a deprivation earlier in the SHRP due to a more developed central nervous system at the time of deprivation.

Secondly, the age to evaluate the deprivation effects determines the observed outcome, at least in rats. For example, 12 months old rats maternally deprived at pnd 3 were hyper-responsive to novelty stress compared to non-deprived littermates, whereas both 3 months old and 30-32 months old rats were hypo-responsive [33]. If these age-dependent responses also hold true for mice we, in our 6 months old mice, might have been testing at the transition point from a hypo- to a hyper-responsive HPA axis, or *vice versa*, from a hyper- to a hyporesponsive HPA axis. Maternal deprivation induced impairment in acquisition of the water maze as observed in 3 and 12 months old rats was not observed anymore in 30-32 months old rats. In the senescent rat, we deal with an age-dependent cognitive decline together with a possible influence of the early life event [18]. However, in a larger subgroup of the maternally deprived 30-32 months old Brown Norway rat we detected a more severe impairment of cognitive function. The finding that mice, like rats, showed more behavioural persistence, thus a decreased flexibility to adapt to a changed environment might be of relevance for cognitive processes of learning and memory. We may argue that increased persistence compensates for a learning deficit. This is what we see in our 6 months old mice. Only examining the effects of maternal deprivation in different age groups will provide an answer to a generalisation of the effects of adverse early life event over species.

Finally, maternal care behaviour expressed by a different degree of licking and grooming of the pups, changes the behavioural and endocrine phenotype of the offspring in later life [8, 10, 17]. It has been shown in rats that early handling intensifies [14] and prenatal stress attenuates maternal care towards pups [1, 15, 32]. In the studies of Oitzl *et al.*, our main comparison regarding rat data, a split-litter deprivation design was used, leaving the hormonal state of the dam and maternal care behaviour undisturbed [18]. In the current mouse study we used a full-litter deprivation. A prolonged separation of the rat dam with her complete litter was shown to evoke compensatory maternal care upon reunion [15]. This deprivation-induced maternal care most likely did not occur in the split-litter deprivation, resulting in more detrimental and longer-lasting effects observed on pituitary-adrenal responsiveness and cognitive performance in the water maze. An alternative explanation lies in the age at deprivation in relation to maternal care. Licking and grooming of high- and low-care rat mothers differs in the first postnatal week, but is comparable four days before the end of the SHRP [14]. Mice were deprived at pnd 8, about four days before the end of the mouse SHRP [24] which makes it likely that maternal care again increased upon reunion.

Summarising, a single 24 hours of maternal deprivation at pnd 8 in mice indeed affected, like in rats, certain aspects of cognitive performance: the flexibility to adapt to a changed environment. However, main effects on learning and long-term memory were not observed. Corticosterone responses to swimming were strong and comparable in maternally deprived and

control mice and might have contributed to the fast acquisition of the spatial learning task. In rats, age-dependent non-linear dynamic changes in cognition and endocrinology were reported. Our study in mice showed that, like in rats, long-term effects most likely depend on the age when maternal deprivation is applied [29, 30, ***Chapter 5***] and on the age of examination in later life [18, 33].

4.6 Acknowledgements

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

Chapter 5

L. Enthoven, E.R. de Kloet & M.S. Oitzl

5.1 Abstract

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

5.2 Introduction

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

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

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

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

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

5.3 Materials and Methods

5.3.1 Animals

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

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

5.3.2 Experimental design

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

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

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

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

5.3.3 Hormone analysis

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

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

5.3.4 In situ *hybridisation*

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

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

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

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

5.3.5 Statistical analysis

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

5.4 Results

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

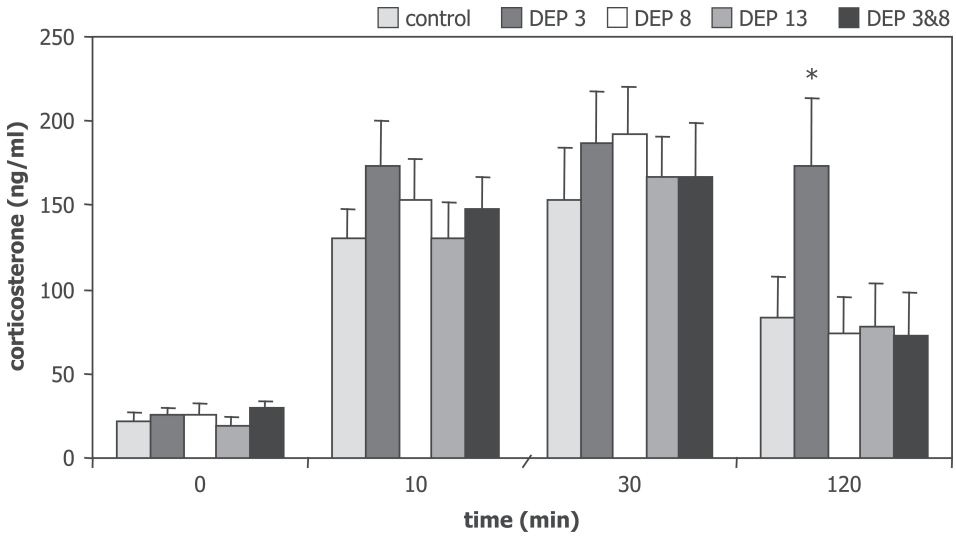

Figure 5.1
Plasma corticosterone concentration (ng/ml) at 28 days of age: basal (t=0 minutes) and at 10, 30 and 120 minutes after exposure to a clean novel cage. Mouse pups had been deprived from their mother for 24 hours once at either pnd 3 (DEP 3), 8 (DEP 8) or 13 (DEP 13); or in a combination of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

5.4.1 Corticosterone (*Figure 5.1*)

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

5.4.2 ACTH (*Figure 5.2*)

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

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

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

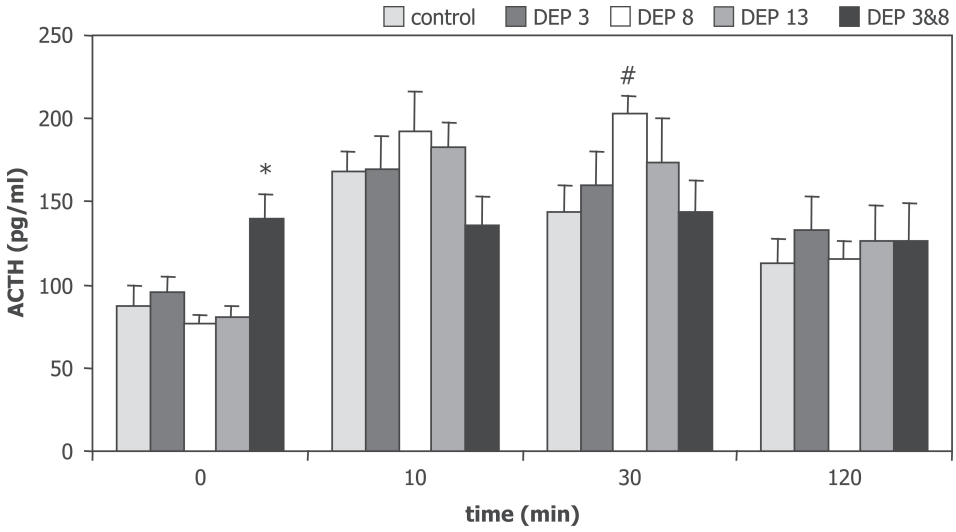

Figure 5.2
Plasma ACTH concentration (pg/ml) in mouse pups at 28 days of age: basal (t=0 minutes) and at 10, 30 and 120 minutes after exposure to a clean novel cage. Mouse pups were deprived from their mother for 24 hours once at either pnd 3 (DEP 3), 8 (DEP 8) or 13 (DEP 13); or in a combination of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

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

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

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

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

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

5.4.4 Body weight (*Table 5.1*)

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

5.5 Discussion

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

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

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

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

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

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

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

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

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

5.5.2 Deprivation outside (pnd 13) the SHRP

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

5.5.3 Repeated deprivation (pnds 3&8)

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

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

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

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

5.5.4 Conclusions

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

5.6 Acknowledgements

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

Chapter 6

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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 Experiment I: 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

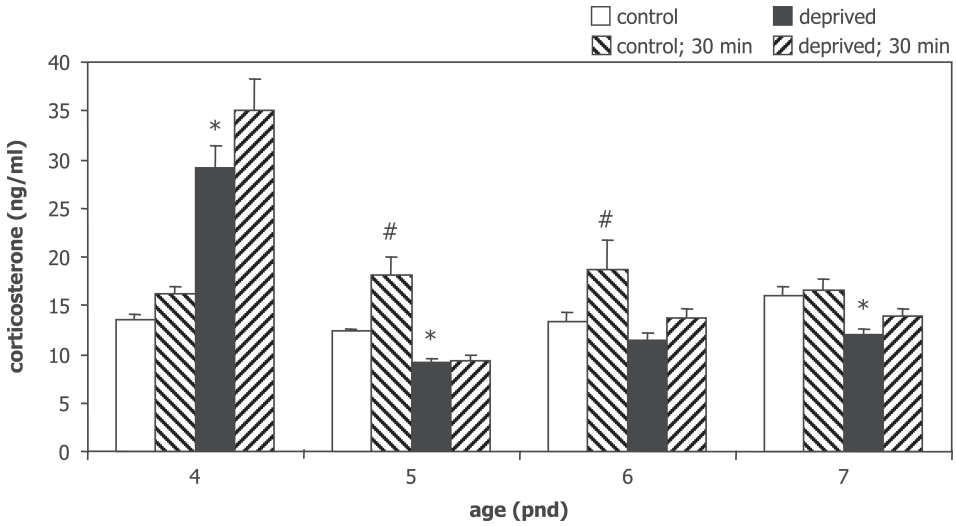

Figure 6.1
Basal and stress-induced plasma corticosterone levels in control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant from basal animals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

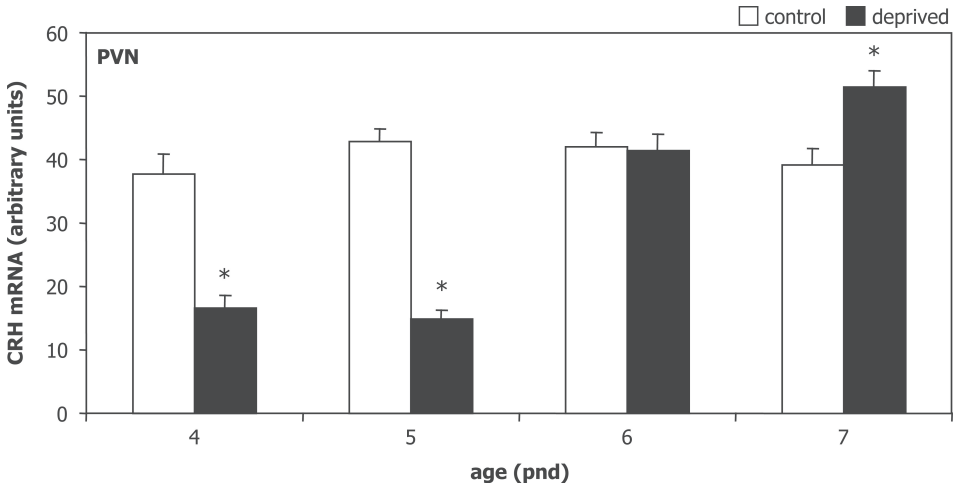

Figure 6.2
Basal expression levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) for control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day.

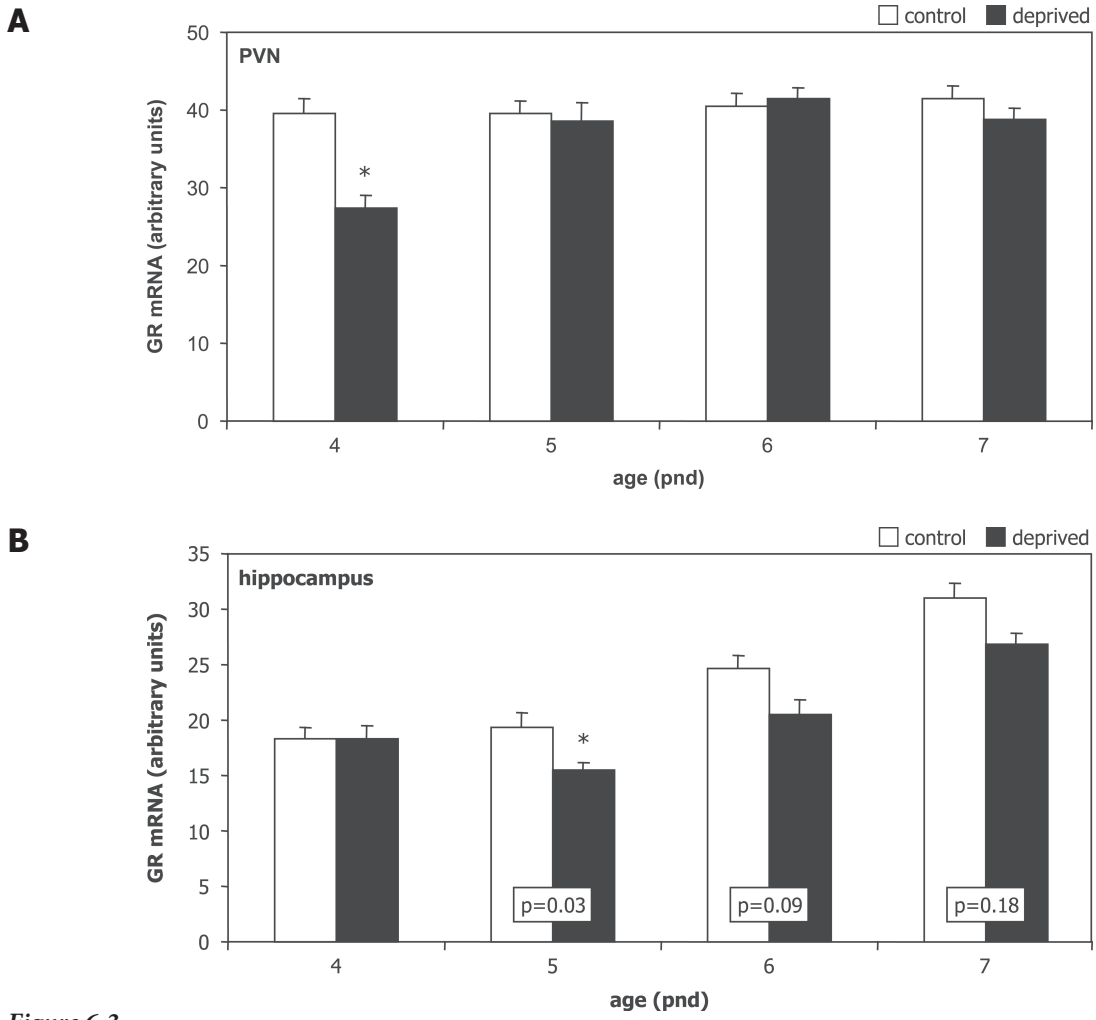

Figure 6.3
Basal expression levels of GR mRNA in the paraventricular nucleus of the hypothalamus (PVN) (***A***) and the CA1 area of the hippocampus (***B***) for control and deprived mouse pups at four postnatal days after maternal deprivation. Data represent mean ± S.E.M., * $P<0.05$ significant from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

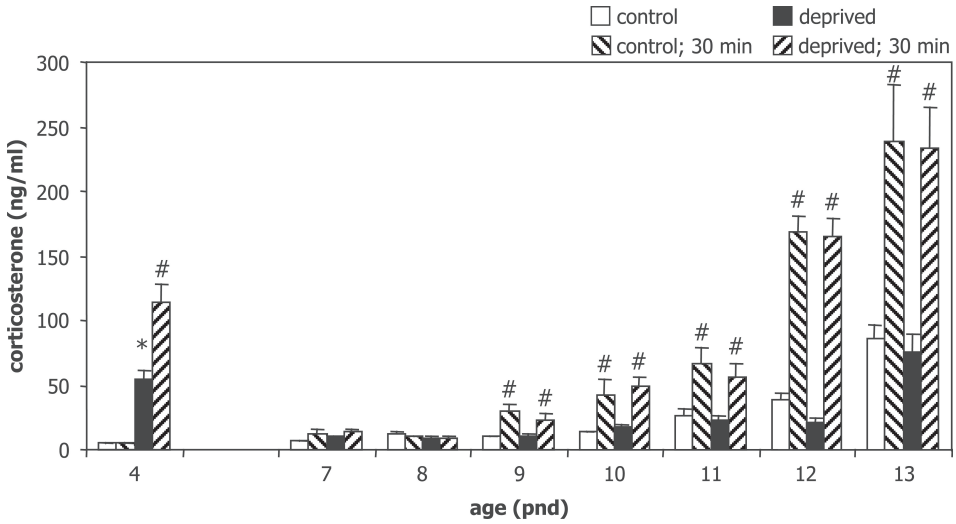

Figure 6.4
Basal and stress-induced plasma corticosterone levels in both control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at pnd 3. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant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

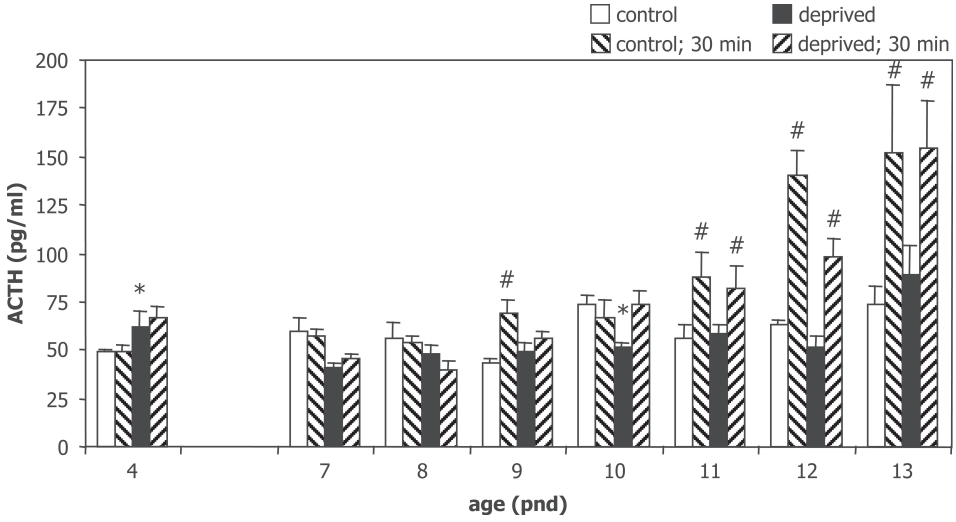

Figure 6.5
Basal and stress-induced plasma ACTH levels in both control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at postnatal day 3. Data represent mean ± S.E.M., * $P<0.05$ significant from control animals at the same day and treatment group, # $P<0.05$ significant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

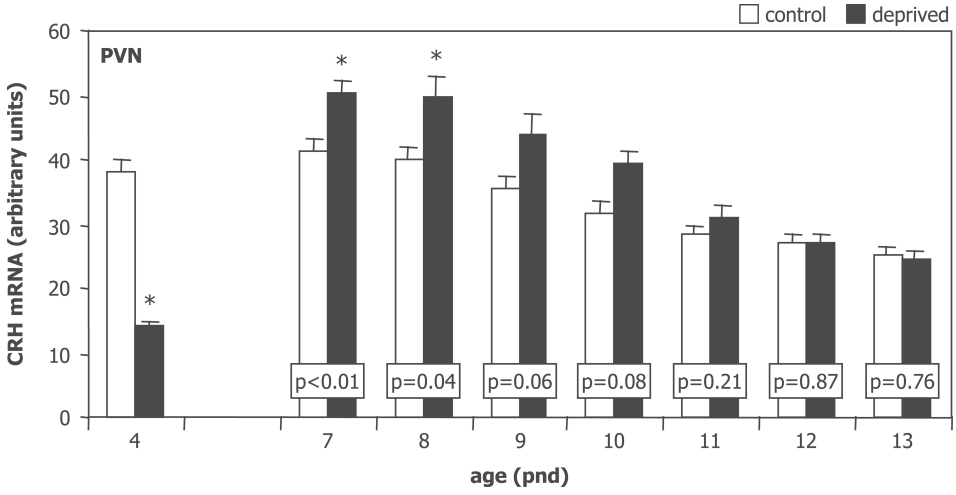

Figure 6.6
Basal expression levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) for control and deprived mouse pups at postnatal days 4 and 7 to 13 after maternal deprivation at postnatal day 3. Data represent mean ± S.E.M., * *P*<0.05 significant from control animals at the same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

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Chapter 7

General discussion

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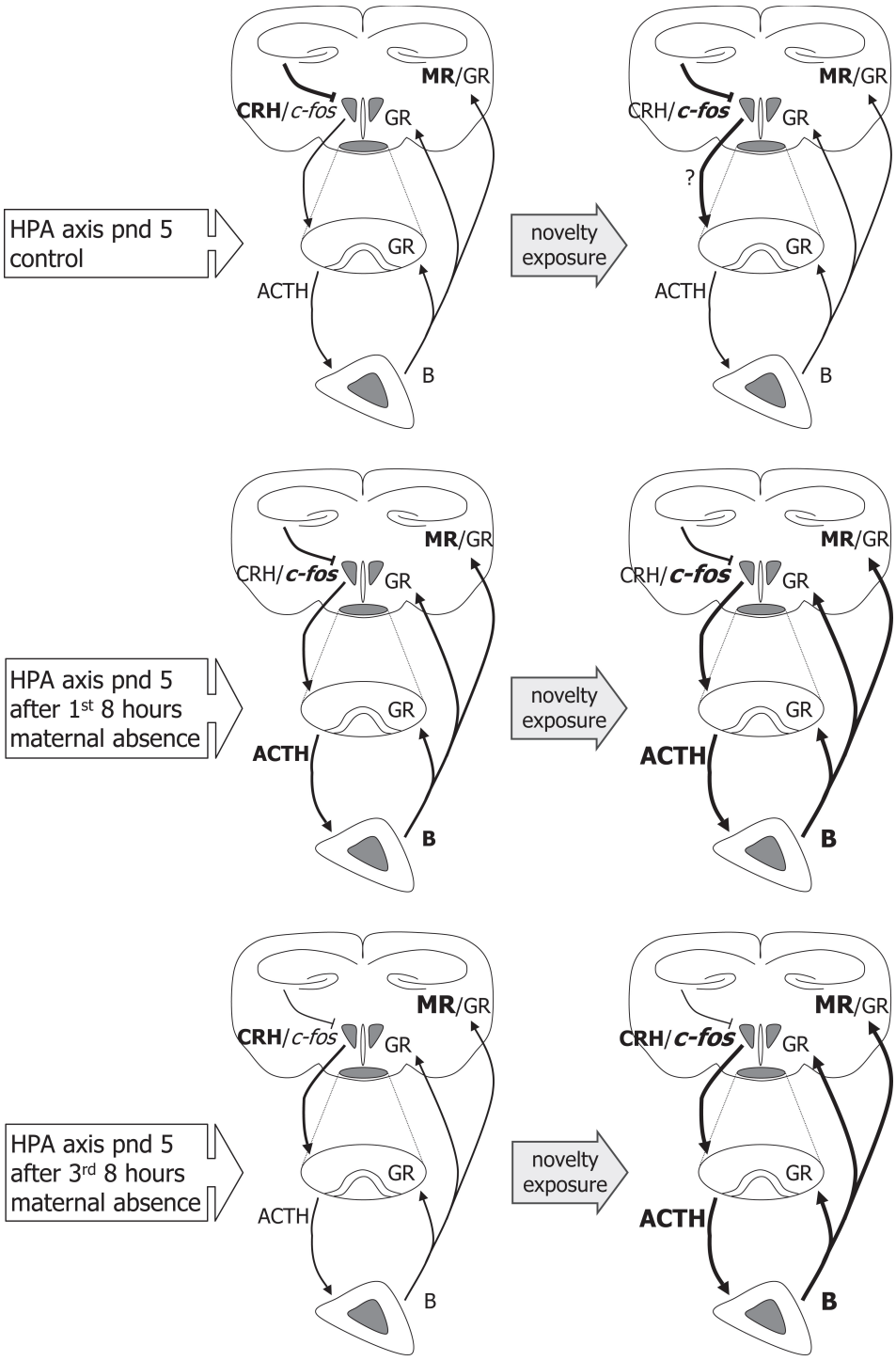
The objectives of the studies presented in this thesis were to examine in mice the immediate and lasting effects of adverse early life experiences on the HPA axis and behaviour. For this purpose two paradigms were used. One paradigm was based on the repeated daily 8 hours separations of CD1 mouse pups from their mother that occurred consecutively on postnatal days (pnd) 3, 4 and 5 (***Chapters 2*** and ***3***). In these experiments the immediate effects of the separations were measured on HPA axis activity with or without additional novelty exposure. The other paradigm was based on a single 24 hours maternal deprivation episode starting at pnd 3 and/or 8 (***Chapters 4*** to ***6***). The developmental effects of this single maternal deprivation were measured at different ages for its consequences for cognitive performance and/or stress responsiveness.

These studies were part of a research program entitled "Nuclear steroid receptors in the brain: target for novel tissue-specific anti-depressants". For this research animal models were needed to study the question how glucocorticoids, which are essential for health, can turn into damaging signals under adverse conditions. Previous studies in the rat had revealed that 24 hours of maternal deprivation created such a vulnerable phenotype [10, 50, 90]. In the program the mouse was investigated, because parallel projects had been carried out with mouse lines genetically selected for aggressive behaviour [86] and for the identification of differentially expressed genes in the hippocampus of these mouse lines [17]. At the time the project started little was known of the outcome of adverse early life experiments in the mouse. Hence, the procedures used for the rat were transferred to the mouse.

Initially, experiments to study long-term consequences of a single 24 hours maternal deprivation on cognition and physiology in the mouse were performed. However, the outcome of this manipulation in the mouse was much more subtle than previously observed in rats [50, 90]. Furthermore, as is also shown in this thesis, the long-term outcome of 24 hours maternal deprivation varied not only as a function of mouse strain and age at deprivation, but also the age at testing appeared important. In order to find clues for these different outcomes it was decided to study more in depth the immediate consequences of maternal separation. For this purpose next to the 24 hours maternal deprivation paradigm we selected the daily repeated (for three consecutive days) 8 hours maternal separations paradigm. At least in rats repeated maternal separations ranging from 15 minutes daily (a procedure called handling) up to 8 hours are commonly used to study long-term effects. In the present study the repeated 8 hours paradigm was selected -in addition to the single 24 hours deprivation paradigm- in the anticipation of robust immediate effects on HPA axis regulation that could serve as basis to understand how the developing brain was shaped towards a vulnerable phenotype in later life.

7.1 Repeated maternal separations

The direct effects of repeated 8 hours maternal separations on HPA axis functioning at pnds 3, 4 and 5 were reported in ***Chapter 2***. The data show that the infant's HPA axis readily desensitises or adapts to the separations, resulting even in reduced basal circulating corticosterone levels.



In spite of this adaptation, the HPA axis remained responsive and the corticosterone secretion became even relatively more sensitised to the effects of novelty exposure. This finding indicates that the repeated maternal separations procedure results in a lasting disruption of the SHRP. In ***Chapter 3*** further experiments are described in search of the underlying mechanism(s) of this adaptation to repeated separations focussing on glucocorticoid feedback. The data show that the change in HPA axis responsiveness is rather of central origin than that it developed due to altered glucocorticoid feedback and/or an altered input of metabolic factors. These data

Figure 7.1 (see opposite page)
Schematic illustration of the different alterations in HPA axis regulation immediately after exposure of neonatal mice to adverse early life events during the stress hypo-responsive period (SHRP). ***Top***: Basal conditions (control) are presented for the undisturbed neonate at postnatal 5 (pnd 5) during the SHRP; ***Middle***: mice separated from their mother for 8 hours (9:00 – 17:00 hours) at pnd 5; ***Bottom***: mice separated from their mother for 8 hours on three consecutive days from pnds 3 to 5. For each condition the response to 30 minutes novelty exposure is presented. Note that after repeated separations, the basal HPA axis activity is similar to that of the undisturbed mice, but in response to novelty the HPA axis now has become responsive.

Bold lines and letters indicate increases in mRNA expression levels (CRH, *c-fos*, MR, GR), circulating hormone levels (ACTH and corticosterone) or excitatory input from limbic structures *e.g.* hippocampal, amygdala, frontal cortex and ascending aminergic pathways to the PVN and from the PVN through the portal vessel system to the pituitary. Larger sized letters indicate more abundant mRNA expression or higher hormone concentrations. ACTH = adrenocorticotropin hormone; B = corticosterone; CRH = corticotrophin-releasing hormone; MR = mineralocorticoid receptor; GR = glucocorticoid receptor. Data is summarised from ***Chapters 2*** and ***3***.

were unexpected as will be pointed out below. They were also quite interesting, because rodents separated repeatedly as infants from maternal care are commonly used as model to study the pathogenesis of depression and other stress-related psychiatric disorders (for reviews see [31, 54, 55, 57, 62]).

7.1.1 Pituitary-adrenal responsiveness

Based on studies performed by Edwards and Burnham [16] and McCormick *et al.* [44], a daily activation of the HPA axis was expected due to prolonged maternal absence. As was hypothesised, such a daily surge in HPA activity would result in episodic exposures of the neonate brain to high levels of corticosterone. Surprisingly, in our experiments each successive period of maternal separation led to a progressively attenuated pituitary-adrenal response to maternal absence until it was completely absent at the third separation period (***Chapter 2***). Hence, at first sight the hypothesis that repeated maternal separation *per se* leads to a cumulative exposure of the brain to corticosterone is not supported under our experimental conditions.

Another remarkable result was that the HPA axis rapidly desensitised 16 hours after reunion. Corticosterone levels were then consistently lower and this state of reduced corticosterone persisted over the next two days. And, even more strikingly, this applies to both the repeated maternal separations (***Chapter 2***) and single 24 hours maternal deprivation model (***Chapter 6***) described in this thesis. Interestingly, these data on reduced glucocorticoid secretion are reminiscent to findings in the human. Hypocortisolism is also observed in children exposed to adverse early life experience [20, 21, 84]. The current studies are therefore the first demonstration that the same phenomenon also can occur in rodents. It raises the intriguing possibility that a 'normal' glucocorticoid level is required for 'normal' brain development and that either a too low or too high level may have detrimental effects.

Interestingly, upon exposure to a novel environment, repeatedly separated pups were still able to elicit a corticosterone response, which seemed even facilitated (***Chapter 2***). A 2-fold induction occurred in animals separated for the first time versus an almost 6-fold induction in repeatedly separated animals. In absolute amounts similar rises in corticosterone concentration were observed. Consequently, the facilitated response to novelty stress in repeatedly separated animals was actually deducted from the lower basal corticosterone values after the third time of

8 hours maternal absence. If 8 hours separation and novelty exposure were combined, it appeared that the decrease in corticosterone truly is an adaptation to repeated maternal absence only. If combined, the third exposure to a novel environment was still a stressful event as judged from the higher corticosterone levels. Thus, within the pituitary-adrenal axis adaptation occured. After 3 days of exposure to repeated maternal separations linked to novelty exposure the ACTH response attenuates in the face of elevated corticosterone, apparently because of increased sensitivity of the adrenal. Since novelty is capable to elicit a corticosterone response in repeatedly separated animals, the SHRP remains permanently disrupted in this paradigm.

Repeated maternal separations can thus produce increased exposure of the brain to corticosterone, if at the same time the animal is exposed to stressors. In this respect, it becomes important how the maternal separation procedure is performed. In the present and some other studies [39, 40] the mother was removed and the pups stayed undisturbed in the same environment for the 8 hours interval. It would be of interest to study the outcome of separations where the pups are removed instead and placed during the 8 hours separation in a novel environment. In fact, in a literature survey it appeared that most studies used the latter paradigm, removing the pups either as a group [43, 47, 53, 58, 59, 87, 89], or individually [32, 44]. If this were the case than indeed the 8 hours removal of the pups may create the necessary conditions to generate an animal model with elevated corticosterone as a result. Such studies performed in systematic fashion are currently being performed in our laboratory [N. Daskalakis, personal communication].

In summary, repeated maternal separations lead to a desensitisation of the infant's HPA axis to the effect of maternal absence and produce a state of persistent hypocorticism. At the same time, the release of the pituitary-adrenal hormones remains highly responsive to novelty, suggesting that in maternal absence the pup's stress system stays on alert. These data indicate that repeated maternal separations can be used as paradigm to study adverse early life events, provided the pup's separation is combined with exposure to a stressful condition.

7.1.2 Central regulation of pituitary-adrenal adaptation

Next, the question of the biological substrate of the HPA axis adaptation to repeated separations was investigated (***Chapter 2***). Since the PVN acts as a central site of integration of information conveyed by neuronal afferents mediating the stress responses [22, 28, 42], the extent of neuronal activation was determined by measuring *c-fos* mRNA expression [7]. In response to a first time maternal absence *c-fos* mRNA expression increased, indicating activation of PVN neurons [74]. However, after repeated separations *c-fos* mRNA expression did not respond to maternal absence anymore. At the same time, a novel environment was able to increase *c-fos* mRNA expression, independent of a first or third period of separation. This reaction pattern of *c-fos* mRNA expression closely resembles therefore that of the pituitary-adrenal responses to repeated separations.

These data clearly show that repeatedly separated pups are able to dissociate between the initial *c-fos* and HPA axis stimulation triggered by maternal separation and the stress-induced

activations due to novelty exposure. Moreover, the subsequent dissociation between these two distinct modes of HPA activation may originate from afferents to the PVN. This is because *c-fos* mRNA expression and corticosterone respond in parallel to repeated separations *per se* with or without additional novelty exposure.

That *c-fos* mRNA in the hypothalamus displayed a corticosterone-like response pattern to repeated separations suggests that a central mechanism is involved in the pup's neuroendocrine adaptation to maternal absence. It may well be that, even though the pups are only 3 to 5 days old, cognitive operations allow to predict that after one experience the mother will return in 8 hours. This would imply that the infant thus rapidly adapts or habituates to an 8 hours period of maternal absence, but that the HPA axis stays on alert and can be activated by stressors. The question of course can be raised if repeated absence perhaps is a more realistic representation of the mouse pup's life, since the mother is frequently away from the nest in the wild [41].

Another line of research is also of interest for the current findings: research by Levine *et al.* [75] and more recently by Moriceau *et al.* [48, 49] has demonstrated that learning can occur in neonatal rats. Infants rapidly learn maternal odor to support attachment behaviour as a function of a locus coeruleus-olfactory bulb pathway during the first week of life and are capable to suppress odor aversions during that time. At the end of the SHRP aversive odors start to activate the locus coeruleus - amygdala circuit fear-motivated avoidance. This switch from maternal attraction to avoidance is facilitated by maternal absence and corticosterone and can be blocked by a glucocorticoid antagonist [48, 49].

In summary, the c-fos *mRNA expression response pattern in the PVN parallels the pituitary-adrenal reactivity after repeated maternal separations. This finding indicates that the desensitisation of the HPA axis to repeated maternal absences is centrally regulated.*

7.1.3 Metabolic aspects

Activation of the HPA axis in early postnatal life is closely linked to metabolic signals. For example, preventing a decrease in glucose or increase in ghrelin levels can block the activation of the HPA axis associated with prolonged maternal absence [70, 82, 83]. Therefore, to investigate an alternative pathway that may contribute to the HPA changes after repeated separations, plasma glucose and ghrelin levels were measured as well as a central target for ghrelin (***Chapter 2***). After repeated maternal separations both glucose and ghrelin still reacted to maternal absence with a response of similar magnitude as when pups were separated from their mother for the first time. As expected, a lack of food for an 8 hours period of maternal absence thus caused a decrease in glucose and a rise in ghrelin, that did not adapt to repeated separations.

Metabolic signals activate cells in the arcuate nucleus [4, 6, 25, 26, 29]. However, in our experiments a direct measurement of neuronal activation of the arcuate nucleus by measuring *c-fos* mRNA expression was unfortunately not possible due to a high non-specific *c-fos* signal originating from the adjoining skull bones. Therefore, we measured NPY mRNA expression in

the arcuate nucleus and CRH mRNA expression in the PVN, a downstream target of arcuate nucleus NPY [4, 6, 25, 26, 29]. These downstream targets of ghrelin were not affected by maternal separations. In view of the metabolism-related data collected so far, there is no evidence that the observed pituitary-adrenal adaptation to repeated maternal separations is due to an altered metabolic signalling. It cannot be excluded, however, that the metabolic state of repeated 8 hours food deprivation, as reflected by the persistent changes in glucose and ghrelin levels, contributes to the enhanced responsiveness of the pituitary-adrenal axis to novelty.

In summary, while it is unlikely that metabolic signals are implicated in desensitisation of the HPA axis under basal conditions after repeated maternal separations, they may contribute to the permanent disruption of the SHRP.

7.1.4 Modulations in corticosterone feedback

The HPA axis hypo-responsiveness during the SRHP is hypothesised to be mediated by aspects of maternal behaviour [23, 30, 60, 77, 78]. The most proximal substrate in maintaining the SHRP is adrenal hypo-sensitivity [60, 77]. As cause of the SHRP enhanced MR- and GR-mediated negative feedback has been proposed [61, 65, 67, 71, 88]. In experiments by Schmidt *et al.* [71] the glucocorticoid antagonist mifepristone administered to 8 days old infants disinhibited the HPA axis, suggesting that indeed GR-mediated suppression of HPA axis activity is one of the determinants of the SHRP. In the present experiments, the GR antagonist did not result in altered pituitary-adrenal responsiveness in mother-reared 5 days old CD1 mice if the animals were maintained under basal stress-free conditions (***Chapter 3***). Blocking the MR with spironolactone resulted in a slight increase in corticosterone, a response that is reminiscent to the MR-mediated control of basal HPA axis activity in adult animals [11, 56, 80].

The inability to trigger an HPA response in 3 and 5 days old pups using a GR antagonist seems at first glance at variance with the finding of Schmidt *et al.* [71]. Differences can be related to differences in pharmacokinetics of solvents used or to an age-related difference in feedback mechanisms. However, this seems unlikely, since the GR antagonist is capable of triggering a profound corticosterone response at pnd 5 after a first time 8 hours maternal separation. Moreover, the MR antagonist, dissolved in the same solvent as the GR antagonist, caused after the first separation a small, but significant reduction in corticosterone secretion, indicating that both antagonists still blocked their respective receptors 8 hours after injection.

Another explanation could be that in Schmidt's experiments there was still some residual HPA activation, which then was targeted by the GR antagonist in order to demonstrate that GR-mediated feedback is an inexorable component to maintain the SHRP. Such residual HPA activations are not uncommon in animals that have been for instance injected with the polyethylene glycol (PEG) solvent of mifepristone. We observed that this particular solvent caused an inflammatory response characterised by elevated IL-6 levels, which very well could have led to the minimal HPA activation needed for GR antagonist disinhibition to become manifest

(***Chapter 3***). If the GR antagonist is administered in saline (NaCl with 0.4% Tween80), as done in the studies described here, the disturbance caused by the injection alone was insufficient to produce a lasting HPA activation of GR-mediated feedback. In short, if there is not even a slight HPA axis activation, a GR antagonist is not expected to demonstrate feedback disinhibition in the same way as previously has been established in the adult [9, 12, 13]. What also can be suggested is that the GR antagonist does not mimic the enhanced HPA axis activation that gradually develops after maternal absence. The GR in neonates does not impose an enhanced suppression of the HPA axis, but, as in adults, GR feedback may only come into place after activation of the stress system to restore homeostasis.

The most striking result was, however, that a GR antagonist was unable to disinhibit the HPA axis in the three times 8 hours separated infants. The hypothesis that the HPA axis has become desensitised due to enhanced glucocorticoid feedback can therefore be rejected. At that time the HPA axis has again become slightly responsive to MR antagonism, which counteracts the reduction in basal levels of corticosterone. This suggests that the central afferents to the PVN underlying HPA axis adaptation to repeated separation have an MR-responsive component.

The two different functional roles of these receptors described for adult animals [9, 12, 13] thus also seem to apply for neonates. When the HPA axis is activated, for instance by exposure to a novel environment or injection of the pup with a systemic stressor, MR antagonism resulted in enhanced secretion of corticosterone and chronic GR antagonism resulted in a prolonged stress-induced corticosterone secretion [11, 56, 71, 80]. On the other hand, blocking MR under basal conditions during the SHRP resulted in slightly elevated basal levels of plasma corticosterone, whereas the GR antagonist did not affect or even slightly reduced basal corticosterone [11, 56, 80]. Our data presented in ***Chapters 3*** thus indicate that also during the SHRP MR-mediated effects are involved in control of basal activity of the HPA axis, whereas glucocorticoid feedback actions are mediated by GR, which contribute to restoration of homeostasis after stress [2, 5, 8, 9, 12, 13]. These data furthermore indicate that, although MR and GR serve important functions controlling HPA axis responsiveness during the SHRP, their relative functions are not different from those observed in adult animals.

To study the role of MR and GR in the HPA axis response to additional novelty stress the antagonists were administered at the end of the separation period. This time point was chosen in order to have, at least for the MR antagonist, equal starting points for each treatment group, because MR blockade was previously found to affect 'basal' pituitary-adrenal levels, thus complicating the interpretation of the results. When pups were repeatedly separated from their mother MR antagonism increased the corticosterone response to novelty further, whereas in untreated animals or in animals separated from their mother for the first time MR antagonism did not affect corticosterone concentrations. This MR-mediated response is comparable to data from adult rats and mice [11, 56]. Blocking the MR therefore relieved the tonic inhibition of the HPA axis [14] and resulted in a faster increase in corticosterone measured shortly after the stressor. Further research is required in order to see whether this increase involves the nuclear

localised MR or the recently discovered membrane MR [27]. Effects of GR antagonism could not be determined under the current conditions because the GR antagonist should then have been administered at the beginning of the 8 hours separation episode [56].

In summary, glucocorticoid feedback can be excluded as cause of the desensitisation of the HPA axis after repeated separations, since the GR antagonist affects the SHRP rather than basal HPA axis activity. A role for MR responsive afferents to the PVN cannot be excluded in particular with respect to the basal activity of the HPA axis, in which MR blockade counteracted the reduction in activity caused by maternal separation.

7.2 Maternal deprivation

As described in the introduction (***Chapter 1***), adult rats display altered cognitive performance and endocrine responsiveness to stress if exposed as pups to a single 24 hours episode of maternal deprivation [10, 33, 34, 50, 90]. It was therefore expected that this paradigm would have a similar outcome in mice. (***Chapters 4*** to ***6***). Below first long-term effects on the HPA axis at either middle age (6 months) or early adolescence (28 days) and then the immediate effects of this procedure are discussed on the HPA development and functionality.

7.2.1 Long-term effects on behaviour and neuroendocrine responsiveness

In ***Chapter 4*** the performance in the water maze of 6 months old mice is described as a consequence of maternal deprivation at pnd 8. Maternal deprivation in mice suppressed the flexibility in the behavioural response of the mice to changes in environmental conditions, whereas the acquisition of the task, in contrast to rats, was not affected [50]. It appeared that during reversal training, mice deprived as pups showed a more persistent search strategy towards the previous platform location before looking for alternatives to escape from the water. A similar lack of flexibility was observed in adult Brown Norway rats deprived as pups from maternal care [50]. Less flexibility or perseverance is considered an advantage as long as the conditions remain the same. Such behaviour becomes maladaptive, however, in a frequently changing environment.

Lasting changes in plasma corticosterone concentrations were expected in the deprived mice, because of the previous rat studies. These studies with the 24 hours deprived Brown Norway rats showed altered corticosterone responses to novelty exposure that varied as function of age. While the control mother-reared rats showed slowly declining corticosterone levels with age, the deprived animals had reduced corticosterone levels at 3 and 24 months. At the same time the hormone response to novelty was strongly enhanced at 12 months [10, 90].

This atypical lifespan pattern in corticosterone was associated with an impaired acquisition of spatial memory [50]. Irrespective of high or low corticosterone levels, spatial learning was impaired in the deprived animals at either age; at senescence, however, in a subgroup of animals only. In fact, at old age a history of adverse early life experience and an atypical lifespan pattern in

corticosterone seemed to drive cognitive performance to the extreme, either excellent and poor learners were identified at the expense of the average performers [50]. Correlates were found in the expression of BDNF in the hippocampus [66]. Alternatively, in human aging continuously elevated corticosteroid levels were predictive for cognitive deterioration [37, 38]. These findings demonstrated that the relationship between lifespan patterns of corticosterone and cognitive performance is complex and requires further investigation.

In contrast to chronically elevated corticosteroid levels, which are damaging and impair cognition, the ability to rapidly switch on and off the corticosteroid response to stress is considered a sign of health. Since the task-related corticosterone responses modulate memory consolidation [12, 45, 63, 64], the time course of the corticosterone response to swimming was determined in the study described in ***Chapter 4***. Maternally deprived and control mice showed a similar response pattern to stressors. Since such rapid corticosterone responses facilitate the formation of spatial memory [14, 63, 76], their similarity in control and deprived mice may explain in part why learning processes were not affected in mice exposed as pups to 24 hours maternal deprivation.

In summary, maternal deprivation at postnatal day 8 reduces cognitive flexibility of 6 months old CD1 mice in a spatial learning task. Formation of spatial memory and the corticosterone response to swim stress were not affected. However, the pattern of changes in corticosterone varies over the lifespan.

7.2.2 Effects dependent on developmental stage

Evidence from rat literature suggest that the age of the pups at which they are separated from their mother provides distinct, but "paradoxical" long-term effects [35, 52, 81]. As described in ***Chapter 5***, maternal deprivation of mice early in the SHRP (pnd 3) resulted in a prolonged corticosterone response to novelty stress at early adolescence (pnd 28), associated with less hippocampal GR mRNA expression. Maternal deprivation late in the SHRP (pnd 8), on the other hand, resulted

Table 7.1: Comparison of the immediate effects of 8 and 24 hours of maternal absence on several HPA axis markers in CD1 mice at pnds 3 and 8.

age:	pnd 3			pnd 8		
hours of maternal absence:	0 hours	8 hours	24 hours	0 hours	8 hours	24 hours
corticosterone	100	400	600	100	450	770
ACTH	100	180	130	100	300	160
CRH mRNA (PVN)	100	100	40	100	70	50
GR mRNA (PVN)	100	100	70	100	n.m.	60
MR mRNA (hippocampus)	100	100	100	100	90	85
GR mRNA (hippocampus)	100	100	100	100	90	85

Values are presented as % compared to the basal levels of undisturbed control mice and calculated for pnds 3 and 8 separately. (*i.e.* >100% means an increase due to maternal absence, <100% means a decrease due to maternal absence, n.m.: not measured) Values are calculated as averages from the data presented in: [69, 72, 73 ***Chapters 2*** and ***6***]. For a detailed explanation of the abbreviations see ***Figure 1.1***.

in an enhanced amplitude of the ACTH response without effects on corticosterone and mRNA expression of central markers of the HPA axis activity. Furthermore, maternal deprivation outside the SHRP (pnd 13) did not affect HPA axis markers.

As will be outlined below, these age-dependent effects could be related to the developmental stage of the brain and the HPA axis at the time of deprivation. The developmental stage depends subsequently on the strain and genetic background of the animal and on the interactions between gene(s) and the environment, in which maternal care is a very important factor [36]. Furthermore, the nature of the adverse experience is of importance, as is its duration and frequency as was shown in ***Chapters 2*** and ***3***.

There are similarities and differences in the direct effects of 8 and 24 hours of maternal absence and between pnd 3 and pnd 8 separated animals (***Table 7.1***) [69, 72, 73, ***Chapters 2*** and ***6***]. The magnitude of the immediate response to separation of corticosterone and ACTH in the 8 days old pup is much larger than in 3 days old pups. Furthermore, maternal absence at pnd 3 has no direct effects on hippocampal MR or GR mRNA expression, while both are downregulated by as much as 15% at pnd 8. Other data from both rats and mice indicate that the response amplitudes of mRNA expression at pnd 3 are the same for CRH and GR in the PVN [15, 72, 73, 85, ***Chapter 6***], but can also be absent for GR and MR in the hippocampus [72, 73, 79, ***Chapter 6***]. Thus, the observation that early or late maternal deprivations culminate in different long-term effects may be considered partly in terms of the developmental state of the brain and HPA axis at the time of maternal absence [68].

Data obtained from rats suggest that the long-term effects of maternal deprivation have an age-dependent pattern, differentially expressed in juvenile, adolescent, adult, aged and senescent animals. In rats early deprivations, between pnds 3 to 5, resulted in enhanced ACTH responsiveness in Sprague Dawley-Long Evens hybrid juveniles (pnd 20 [81]), a prolonged corticosterone response at 2-3 months of age in Long Evans rats [52] or a reduced one in Brown Norway rats of the same age [90], an enhanced response in Brown Norways at 6 months of age [90], no effect in 20 months old Wistar rats [35] or reduced again in Brown Norway rats [90]. Late deprivations, between pnds 9 to 14, resulted in these strains in an attenuated ACTH responsiveness in juveniles [81], an enhanced ACTH response in adults [52] or remained again without effect in aged animals [35]. We have measured the effects of a late deprivation (pnd 8) at two ages and observed an enhanced ACTH response in early adolescent mice (pnd 28, ***Chapter 5***) and no effect in adults (6 months of age; ***Chapter 4***). This is in contrast to rat data and indicates that indeed next to an age effect also species and strain differences, as demonstrated in rats, complicate the interpretation of the effects of adverse early life events.

In summary, lasting effects of a single maternal deprivation episode on HPA axis regulation appear not only to dependent on the age of the infant and the duration of the separation, but also on species and strain differences. In response to an adverse early life event the outcome in later life is neither

monolithic nor linear, but rather concerns an altered pattern of lifespan changes in the HPA axis.

7.2.3 Consequences for HPA axis development

In rats handling accelerated the maturation towards adult-like circadian corticosterone rhythmicity [1]. Whether in mice maternal separation also affects the rate of maturation of the HPA axis was studied in ***Chapter 6***. The goal was to investigate the precise developmental pattern of the HPA axis towards emergence from the SRHP after maternal deprivation at pnd 3.

As described in ***section 7.2.2*** and displayed in ***Table 7.1*** 24 hours of maternal absence during the SHRP had robust direct effects, elevating basal corticosterone and ACTH values and rendering the HPA axis responsive for mild stimuli. Strikingly, after reunion of the pups with their mother, both basal and stress-induced ACTH and corticosterone were reduced for at least three days (***Chapter 6***). Interestingly, this reduction was also observed for the repeated maternal separations paradigm (***Chapter 2***). Thereafter, these measures in the maternally deprived pups were indistinguishable from control animals and both groups emerged from the SHRP at the same time, gradually from pnd 9 onwards. Recovery of the central components of the HPA axis proceeded, however, in different time domains. CRH mRNA expression remained at 50% for 2 days (pnd 4 and 5) and thereafter was higher than in control animals until pnd 11, when it caught up with the normal decreasing developmental pattern. In previous studies GR mRNA expression in the PVN was reduced in response to maternal absence, but returned to control levels already 24 hours after reunion. [3, 16, 72, 73, 79]. In contrast, GR mRNA expression in the hippocampus of the 3 days old mouse pup showed an arrest in GR mRNA expression development until pnd 5, where after expression gradually increased towards control levels. MR mRNA expression, on the other hand, remained unaffected. How these temporal changes in the various brain areas are linked remains to be investigated.

Thus, a single 24 hours maternal deprivation did not result in a permanent disruption of the hypo-responsiveness of the HPA axis. In this respect the maternal behaviour received by pups upon reunion is an important factor in the development of the stress system [19, 24, 46]. Maternal care behaviour is most strongly expressed during the first ten postnatal days [40]. Nursing and licking of the pups are positively correlated to stress coping, *i.e.* if more nursing and licking occurred in early life, this results in lower ACTH and corticosterone responsiveness to stress as adults [46]. One of the best known examples comes from studies demonstrating that daily handling triggers increased maternal care upon reunion [18]. In addition, pups that were separated for 4 hours from their mother as a whole litter evoked a compensatory bout of maternal care [40]. Furthermore, maternal care is an interactive process. For example, older pups are able to elicit more nursing and licking than younger pups [75]. CD1 mice, the strain used in the experiments described in this thesis, show at adulthood relatively low anxiety [51]. In rats this low anxiety is correlated to high licking and grooming behaviour of the mother [46]. Thus, the observed gradual return of most HPA axis markers that were affected by maternal deprivation could be in part consequential of the maternal care received by the pups.

In summary, the profound activation of the infant's HPA axis by 24 hours of maternal deprivation is followed by a transiently lower basal activity lasting at least 3 days for ACTH, corticosterone and CRH mRNA expression. The various HPA axis markers return gradually and in individually different time domains to a developmental pattern indistinguishable from that of controls. To what extent these temporal changes depend on maternal care upon reunion remains to be established.

7.3 Implications of the findings

In retrospect the studies in this thesis have a number of interesting implications, which will first be discussed from a developmental perspective and then in view of their significance for the development of an animal model for psychiatric disorders.

7.3.1 Developmental perspective

It can be concluded that the current studies demonstrate an excelent example of the amazing plasticity of the infant mouse's brain in a changing environment. Hallmarks are, in response to repeated separations, the rapid desensitisation of the HPA axis, the persistently lower basal HPA axis activity and the permanent nature of SHRP disruption. These features were noted after a three times repeated daily 8 hours separation. In some aspects, *i.e.* downregulation of basal HPA activity and disruption of HPA axis, this model resembles the impact of a single 24 hours deprivation. However, in response to maternal absence, the two models are completely different. A single 8 hours separation or a 24 hours deprivation sensitises the HPA axis to novelty, while repeated separations result in the opposite: desensitisation. Metabolic factors and glucocorticoid feedback are unlikely factors determining the desensitisation of the HPA axis to repeated separations, but a role of a central MR-responsive network activating *c-fos* expression in the PVN cannot be excluded. The cause underlying the persistent disruption of the HPA axis remains elusive and requires further experiments.

The question can be raised how representative the current laboratory conditions of daily 8 hours are for the rodents living in the wild. As was pointed out by Macri and Würbel [41] such pups live in the safe and stable environments of the nest from which the mother is away many times to secure food. In the current experiments the pups were maintained in the same environment. Does this imply that desensitisation of the HPA axis and disruption of the SHRP actually is the natural condition for rodents? If so, this could mean that the continuous close proximity of mother and pup is a special feature characteristic for laboratory conditions. And alternatively, it may imply that maternal absence, which is usually indicated as an adverse condition, actually is daily life in the wild. It also raises the issue of the significance of maternal care upon return of the mother to the nest.

7.3.2 Significance for animal model

The experiments described in this thesis also have demonstrated the complexity of generating a vulnerable phenotype after a traumatic early life experience, since the outcome of this experience is

largely determined by maternal care. In fact, there exist strong correlates between the high quality of maternal care and enhanced cognitive performance, a large long-term potentiation response, increased length of apical dendrites and increased number synaptic boutons [D. Champagne, personal communication]. However, our maternal separation experiments clearly demonstrate that maternal care is not the only factor involved, as was also put forward by Würbel [41]. Strain and gender, as well as time and duration of the separation and the amount of maternal care received upon reunion all are significant factors determining outcome. Collectively, all these factors contribute to the profound individual variation in coping with stress, aspects of motivational behaviour and cognitive performance and these subtle methodological differences may be crucial for understanding the mechanisms underlying the lasting effects of early adversity.

7.4 Conclusions

The studies presented in this thesis focussed on the consequences of adverse early life experiences for the development of the HPA axis in the newborn mouse. Both the immediate effects of repeated 8 hours maternal separations, as well as the developmental effects of a single 24 hours maternal deprivation were studied. Based on our results the following conclusions can be drawn:

1. After 8 hours of maternal absence mouse pups mount a profound pituitary-adrenal response, which is abolished when the separation is repeated the next days. This desensitisation is accompanied by a lower basal HPA axis activity. The infant readily adapts to repeated maternal absence, possibly because the pup has learned to predict the upcoming return of the dam.
2. While the infant's HPA axis adapts to daily maternal absence, the stress response system stays on alert. The SHRP therefore remains disrupted and mild stressors, such as the exposure to a novel environment, can still trigger a profound HPA axis response.
3. The adaptations in pituitary-adrenal activity achieved by repeated maternal separations seem to depend on changes in central MR-responsive stimulatory afferents triggering a *c-fos* response in the PVN rather than on changes in GR-mediated feedback or metabolic inputs. Whether the persistent disruption of the SHRP due to repeated maternal separations depends on GR in this model still needs to be firmly established.
4. 24 hours of maternal deprivation early in the SHRP (pnd 3) causes a profound suppression of various HPA axis parameters that last several says and then return to control levels in different time domains. However, the emergence from the SHRP is not different from undisturbed animals.
5. The immediate and long-term outcome of a single 24 hours maternal deprivation depends on age and genetic background as well as on duration and frequency of this adverse event. Moreover, the long-term outcome varies over the lifespan.

7.5 References

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Summary
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Summary

Adverse early life events in humans have the capability to change the susceptibility for stressful events later in life. Early life adversity is therefore presented as a risk factor in those subjects, who already have a genetic predisposition to develop stress-related psychiatric disorders. The developmental constraints that underlie the lasting consequences of these early life events are, however, poorly understood. Therefore, the separation of rodent infants from their mother is frequently used as a laboratory model to study both the immediate effects and its long-term outcomes.

The studies presented in this thesis focus on the hypothalamic-pituitary-adrenal axis (HPA axis). In ***Chapter 1*** a description is given of the function of the adult HPA axis and its main components. This is followed by a section in which the role of the HPA axis in the development of a vulnerable phenotype for affective psychopathology is presented, as well as the implications of genotype-environment interactions. Both human epidemiological and animal experimental studies have shown that early (social) experiences influence the functioning of physiological processes even into adulthood. To understand the mechanisms underlying the effects of adverse early life events and its effects on HPA axis functioning it is imminent to have knowledge on the development of the HPA axis and on factors that regulate this development.

Chapter 1 continues with a description of the characteristics of the rodent stress hypo-responsive period (SHRP) lasting in mice from postnatal day 1-12. During the SHRP mild stressors, such as exposure to a novel environment, trigger only a very weak pituitary ACTH and adrenal corticosterone response, which is profound under the same conditions in the adult animal. At that time the HPA axis still develops and has not reached adult functionality yet. Furthermore, the SHRP is hypothesised to be a critical period in rodent development, in which environmental factors can 'program', among others, the responsiveness of the HPA axis. Therefore, the current ideas on mechanisms underlying the SHRP are presented. The extrapolation of SHRP knowledge to man is then discussed to evaluate the validity of animal models. Though numerous models are currently described in literature, the focus in this thesis was on two models: repeated daily 8 hours maternal separations and a single 24 hours maternal deprivation. Interestingly, in the case of repeated maternal separations most data in literature refer to long-term effects, whereas using the 24 hour maternal deprivation paradigm mostly the immediate effects have been examined.

The ***objectives*** of the studies presented in this thesis are therefore to test the following two hypotheses:

- Mouse pups exposed to repeated daily 8 hours separations from maternal care display as a result of this experience a sustained activation of the HPA axis during the SHRP.
- Mouse pups tested in the single 24 hours maternal deprivation model display an altered developmental trajectory of the HPA axis and have as adults long-term changes in cognitive performance and HPA responsiveness to stressors.

In ***Chapter 2*** we examined whether repeated daily bouts of 8 hours maternal separation, in the absence or presence of an additional novelty stressor, would result in a persistent elevation of corticosterone in CD1 mice. For this purpose the effects of repeated 8 hours separations from postnatal days 3 to 5 were measured on basal and stress-induced ACTH and corticosterone concentrations. Furthermore, mRNA expression of selected HPA axis markers and of *c-fos* in the brain and circulating levels of glucose and ghrelin, including their hypothalamic NPY mRNA endpoint, were also measured.

The results showed that the infant's initial immediate HPA axis response to 8 hours of maternal absence was gradually eliminated when the maternal separations were repeated the next two days. Despite the absence of an HPA axis response to the third maternal separation at postnatal day 5, these mouse pups continued to respond to novelty exposure (*i.e.* subjecting a mouse pup individually to a clean novel cage). Interestingly, when the repeated maternal separations were each time combined with novelty exposure, the response of corticosterone secretion relative to ACTH was enhanced. In addition, these effects of separation on the HPA axis activation were reflected by the *c-fos* mRNA expression pattern in the PVN, but not in cortex or thalamus. *C-fos* mRNA expression in the PVN showed a profound response to a single separation and subsequent desensitisation to repeated separations, while remaining responsive to novelty exposure. Basal circulating levels of corticosterone and ACTH were persistently suppressed 16 hours after reunion, an effect that also transiently occurred for CRH mRNA in the PVN. Pituitary POMC and GR mRNA expression in hippocampus or hypothalamus did not change. However, MR mRNA expression in gyrus dentatus of the hippocampus showed a progressive increase with repeated separations, surpassing the gradual increase observed in undisturbed mother-reared mice. Circulating levels of ghrelin and glucose, indicative for depleting energy substrates, and their hypothalamic NPY mRNA endpoint did not match the maternal separation-induced corticosterone patterns in the infant. Glucose levels decreased and ghrelin levels increased after the first as well as the third maternal separation. These data clearly indicate that, while the mouse pup's initial HPA axis response to repeated maternal absence readily desensitised and a state of hypocorticism was induced, the HPA axis did remain highly responsive to mild stressors, indicating a permanent disruption of the SHRP.

Chaper 3 elaborated on the data observed in ***Chapter 2*** and determined whether the neuroendocrine desensitisation to maternal absence as observed and described in the previous chapter is due to enhanced glucocorticoid feedback. For this purpose the effects of either MR or GR antagonists were measured on circulating corticosterone levels. The results indicated that when the GR antagonist mifepristone was applied at postnatal day 5, the infant's corticosterone response to the first separation was amplified. However, the GR antagonist became ineffective if the pups were exposed to repeated maternal absence in the preceding two days. Under basal conditions during the SHRP (at postnatal days 5 and 8) the GR antagonist caused generally a small decrease rather than an increase of circulating corticosterone levels. However, upon a subtle HPA activation, achieved by a mild immune challenge, the GR antagonist became effective again

in interfering with glucocorticoid feedback and then thus triggered a profound corticosterone increase. Blockade of the MR by spironolactone resulted in small but significant increases in circulating corticosterone levels under basal conditions after 8 and 24 hours, as well as after the third separation. MR antagonism did not increase, but rather decreased corticosterone levels when the animals were separated for the first time. During novelty exposure a brief exposure to the MR antagonist enhanced the corticosterone response both in the single and repeatedly separated pups, while GR antagonism under those short-term conditions was ineffective.

In conclusion, these findings excluded an enhanced glucocorticoid feedback as mechanism underlying the desensitisation of the HPA axis in response to repeated maternal separations and support the role of the GR in maintenance of the SHRP. The results also indicate the operation of an MR-responsive mechanism during the SHRP that mostly restrains HPA activity under basal, stressful and maternal separation conditions. The current evidence thus points towards the implication of an MR-responsive central PVN input that abolishes the pituitary-adrenal response to repeated maternal absence, but maintains the neuroendocrine responsiveness to novelty.

In the second part of this thesis the long-term effects of a single 24 hours maternal deprivation were determined. In ***Chapter 4*** it was first investigated if 6 months old CD1 mice subjected as infants to a single 24 hours maternal deprivation at postnatal day 8 showed modulated cognitive performance in a spatial learning task and had an altered HPA axis response to a stressful event. It was observed that maternal deprivation indeed affected reversal learning in the water maze, indicating that a more persistent and less flexible behaviour had developed. However, acquisition and long-term memory were not affected. This implied that the long-term effects of maternal deprivation in mice are subtle. The endocrine response to stress was not affected. In view of current knowledge the results support the notion that, like in rats, long-term effects depend on the age at which the maternal deprivation is experienced, while also the age at examination is important.

In ***Chapter 5*** the effects of maternal deprivation at different ages during the SHRP on endocrine responsiveness and basal mRNA expression of selected HPA axis markers at early adolescence (weaning; postnatal day 28) were compared in order to gain more insight into the observed effects of maternal deprivation described in ***Chapter 4***. It was observed that maternal deprivation did produce long-term changes in the HPA axis, both at the basal set point and in responsiveness to a mild stressor, but only when the deprivation period was applied within the SHRP. Early in the SHRP (postnatal day 3) maternal deprivation prolonged the corticosterone response to stress at weaning and reduced basal GR mRNA expression in the CA1 subfield of the hippocampus, whereas at a later age (postnatal day 8) maternal deprivation only enhanced the ACTH response. Strikingly, a subsequent deprivation (postnatal days 3 and 8) interfered with the effects induced by the first deprivation (postnatal day 3), resulting in sustained, non-responsive high ACTH concentrations and concomitantly a corticosterone response to stress, which was indistinguishable from that in control animals. These data thus further underscore

the importance of the pup's age at deprivation and, consequently, the developmental stage of the brain and the HPA axis. These data add to the current view that the outcome depends on age of the pup and the nature, duration and frequency at which the adverse event is experienced, while sex and genetic background are also of crucial importance.

In ***Chapter 6*** several HPA axis markers were investigated during SHRP development after maternal deprivation at postnatal day 3. This study was initiated to test the hypothesis that maternal deprivation will alter the subsequent developmental pattern of the HPA axis. It was shown that the short-term effects partly resemble those observed for maternal deprivation at postnatal day 8, implying an enhanced basal and stress-induced ACTH and corticosterone concentrations, as well as a reduction of CRH and GR mRNA in the PVN. Differences were the lack of effects on hippocampal MR (all regions) and GR mRNA expression (CA1 region), which actually showed a delay in development. One day after reunion of the pups with the dam the SHRP was reinstated. A novelty stressor was no longer able to activate the HPA axis in previously deprived mice; basal ACTH and corticosterone were suppressed to levels below control for three days. Maternal deprivation did not influence the duration of the SHRP, as determined by the novelty-induced corticosterone and ACTH. In addition, most of the robust short-term effects on central HPA axis markers gradually re-adjusted in the days following reunion, returning to control levels each at its own rate. GR mRNA in the PVN was reduced for only one day before returning to control levels. However, CRH mRNA in the PVN was suppressed for two days, but exceeded control levels at postnatal day 7 after which it gradually declined and catched up again to the declining control levels. So, the robust deprivation effects on various HPA axis markers gradually recovered after reunion and maternal deprivation early in the SHRP did not influence the duration of the SHRP.

The results described in this thesis were discussed in a broader perspective in ***Chapter 7***. To illustrate ***Figure 7.1*** gives a schematic overview of the different alterations in HPA axis regulation when neonate mice are subjected to a single or repeated 8 hours maternal separations. The results have led to the concept that mouse pups readily adapt to repeated maternal absence through a presumed cognitive mechanism that allows processing of novel stimuli into a physiological stress response at times mother-reared animals remain quiescent under the same stressful conditions. Regarding the outcome of a single adverse event of 24 hours of maternal deprivation a bewildering variation in immediate effects and long-term consequences is noted that depend on the context or developmental stage of the brain, the genetic background and the age during lifespan the individual is being tested.

In conclusion:

- The mouse pup's HPA axis readily adapts to repeated daily maternal absence for 8 hours, but remains highly responsive to other stressors.
- Central cognitive inputs rather than peripheral metabolic inputs seem to facilitate the

observed adaptation to repeated maternal absence.

- In response to 24 hours of maternal deprivation at postnatal day 3 the various HPA components gradually return, after initial activation, to the values observed for the mother-reared animals to conclude with similar emergence from the SHRP.
- The outcome of 24 hours of maternal deprivation varies over the lifespan and depends on the age and genetic background of the infant, as well as on the duration and frequency of the adverse event.

Samenvatting

Een traumatische vroege levenservaring kan de wijze waarop mensen later in het leven omgaan met stressvolle gebeurtenissen beïnvloeden. Zo'n vroege levenservaring wordt daarom ook wel gezien als een risicofactor voor stressgerelateerde psychiatrische ziekten, zoals depressie in individuen met een genetische aanleg hiervoor. Het mechanisme, dat ten grondslag ligt aan de blijvende gevolgen van vroege levenservaringen, is echter nauwelijks bekend. Wel is bekend, dat voor de ontwikkeling van de hersenen en het stress systeem de binding aan de moeder van uitermate groot belang is. De scheiding van de moeder is dan ook een veelgebruikt laboratorium model voor verwaarlozing. Het hier beschreven onderzoek aan de muis maakt dan ook gebruik van de scheiding van moeder en pup om zowel de onmiddellijke gevolgen als die op langere termijn te onderzoeken.

Het onderzoek is gericht op het effect van de onthouding van moederzorg op de ontwikkeling van de hypothalamus-hypofyse-bijnier (HHB) as (Engels: hypothalamic-pituitary-adrenal (HPA) axis). Deze HHB as is cruciaal voor de omgang met stress en wordt in detail beschreven in ***Hoofdstuk 1***. Zowel fysieke als psychische stressoren kunnen de HHB as activeren; de paraventriculaire nucleus van de hypothalamus (PVN) geeft corticotropine-releasing hormoon (CRH) en arginine vasopressine (AVP) af aan de eminentia mediana waar het portaalvatensysteem de neurohormonen opneemt en onmiddellijk naar hypofysevoorkwab voert. Hier stimuleren deze hormonen vervolgens de afgifte van adrenocorticotroop hormoon (ACTH) in de bloedsomloop. ACTH stimuleert op zijn beurt de productie van corticosteron in de bijnierschors. De activiteit van de HHB as wordt via een negatief terugkoppelingsmechanisme geregeld en hiertoe bindt corticosteron aan de glucocorticoid- en mineralocorticoidreceptoren (GR en MR). Deze negatieve terugkoppeling verloopt in de HHB as via GR in de PVN en de hypofyse. Daarnaast koppelt corticosteron ook terug op die processen, die in oorsprong de HHB as hebben geactiveerd. Deze stressreacties kunnen ontstaan zijn door een verstoring van het energiemetabolisme en door immuun- en ontstekingsreacties. Corticosteron voorkomt dan dat deze stressreacties, die essentieel zijn voor de verdediging van het lichaam, doorschieten en zelf schadelijk worden. Psychosociale stressreacties zijn de grootste verstoorders van het evenwicht in lichaam en geest (ook wel homeostase genoemd). Bij het herstel van homeostase na psychische stress zijn zowel MR als GR betrokken. Die brengen gedragsaanpassingen tot stand en hebben een belangrijke functie in emotie, motivatie en gemoedstoestand.

Epidemiologisch onderzoek heeft uitgewezen, dat vroege (sociale) ervaringen het functioneren van fysiologische processen zelfs tot in volwassenheid kunnen beïnvloeden. Een vergelijkbaar resultaat is geboekt in dierexperimenteel onderzoek met ratten en muizen en ook met primaten. Het is bekend dat de HHB as een belangrijke rol speelt in het ontstaan en verloop van psychiatrische aandoeningen. Om het mechanisme te kunnen begrijpen, dat ten grondslag ligt aan deze levenslange effecten van vroege ervaringen, is inzicht in de (normale) ontwikkeling

van de HHB as van groot belang.

Hoofdstuk 1 gaat daarom verder met een beschrijving van de kenmerken van de stress hyporesponsieve periode (SHRP), die in de muis duurt van dag 1 tot 12 na de geboorte (postnataal). Tijdens de SHRP kunnen milde stressoren (novelty stressor genoemd; zoals blootstelling aan een nieuwe omgeving) slechts een zeer geringe ACTH- en corticosteronrespons veroorzaken, terwijl deze respons aanzienlijk is in volwassen dieren bij dezelfde stressor. Gedurende de SHRP ontwikkelt de HHB as zich, maar heeft nog niet dezelfde functionaliteit bereikt, zoals die zich bij volwassen dieren manifesteert. Bovendien wordt deze periode over het algemeen gezien als een kritieke periode in knaagdierontwikkeling, waarin omgevingsfactoren en dus ook traumatische levenservaringen de responsiviteit van (onder andere) van de HHB as blijvend kunnen wijzigen. Recent is ontdekt dat als onderdeel van deze 'programmering' de expressie van de GR blijvend veranderd kan worden via epigenetische processen. Deze epigenetische processen worden in dit proefschrift verder buiten beschouwing gelaten.

De extrapolatie van de ideeën over de SHRP in knaagdieren naar mensen wordt besproken ten einde de betrouwbaarheid van diermodellen te kunnen vaststellen. Er zijn vele verschillende diermodellen beschreven, die alle variaties op het thema 'scheiding van moeder en pup' betreffen. In dit proefschrift zijn twee modellen toegepast in CD1 muizen: (1) een driemaal herhaalde dagelijkse maternale separatie van 8 uur en (2) een enkele maternale deprivatie van 24 uur. Bij de herhaalde maternale separaties richten we ons op de directe effecten (***Hoofdstukken 2*** en ***3***), omdat er tot nu toe voornamelijk lange termijn effecten beschreven zijn zonder dat bekend is hoe die effecten tot stand komen. Voor de enkele maternale deprivatie is er juist vooral veel bekend over de directe effecten en zijn de gevolgen voor de lange termijn onderzocht (***Hoofdstukken 4, 5*** en ***6***).

De ***doelstelling*** van het onderzoek in dit proefschrift betreft het toetsen van de volgende hypothesen:

- Pups, die blootgesteld worden aan herhaalde dagelijkse separatie van maternale zorg voor periodes van 8 uur, hebben als gevolg van deze ervaring een permanent geactiveerde HHB as tijdens de SHRP.
- Pups, die blootgesteld worden aan een enkele episode van 24 uur maternale deprivatie, laten veranderingen zien in de ontwikkeling van de HHB as, waardoor ze als volwassen dieren anders reageren tijdens leertaken en tevens een veranderde HHB as responsiviteit voor stressoren laten zien.

In ***Hoofdstuk 2*** werd onderzocht of dagelijks herhaalde perioden van 8 uur afwezigheid van de moeder van het nest (maternale separatie) op postnatale dagen 3, 4 en 5 in de aan- of afwezigheid van een extra novelty stressor, namelijk het plaatsen van de pups in een schone, nieuwe kooi, leidt tot een continu verhoogd corticosteronniveau in CD1 muizenpups. Naast de effecten van herhaalde 8-urige separaties op basale en stress-geïnduceerde ACTH- en corticosteronconcen-

traties in het bloed, werd ook de mRNA expressie van geselecteerde HHB as markers en van *c-fos* in hersencoupes bepaald. Tevens werden de bloedplasmaconcentraties van glucose en ghreline gemeten, inclusief hun hypothalamische NPY mRNA eindpunt.

Uit de resultaten bleek, dat de initiële onmiddellijke HHB as respons op 8 uur maternale afwezigheid bij de pups geleidelijk verdween als de maternale separatie de twee volgende dagen werd herhaald. Ondanks de afwezigheid van een HHB as respons op de derde maternale separatie (op postnatale dag 5), reageerden de muizenpups echter nog steeds op een novelty stressor. Wanneer de herhaalde maternale separaties werden gecombineerd met deze novelty stressor werd de corticosteronrespons zelfs versterkt in vergelijking met de waargenomen ACTH-respons. Deze separatie-effecten op de HHB as werden ook teruggevonden in het *c-fos* mRNA expressiepatroon in de PVN, maar niet in de cortex of thalamus. Na de eerste maternale separatie werd een flinke respons op *c-fos* mRNA expressie gezien en vervolgens een desensitisatie na herhaalde separaties, terwijl de responsiviteit op een novelty stressor bleef. Basale concentraties van corticosteron en ACTH in het bloed werden onderdrukt tot 16 uur na herening met de moeder, een effect dat ook optrad voor CRH mRNA expressie in de PVN. De mRNA expressie van hypofyse POMC en GR in de hippocampus of hypothalamus veranderden niet. MR mRNA in de gyrus dentatus van de hippocampus liet echter een continue stijging zien naargelang meer separaties hadden plaatsgevonden, welke zelfs de normale geleidelijke stijging waargenomen in ongestoorde pups voorbij ging. Bloedplasmaconcentraties van ghreline en glucose, indicatief voor een veranderd patroon in de energiehuishouding, en hun NPY mRNA eindpunt kwamen niet overeen met het door maternale separatie geïnduceerde corticosteronpatroon in de pup. Glucoseniveaus daalden en ghrelineniveaus stegen, zowel na de eerste als na de derde maternale separatie. De metabole signalen veroorzaken dus niet de waargenomen HHB aanpassingen.

Deze data laten duidelijk zien dat, terwijl de pup's initiële HHB as respons op herhaalde maternale separaties desensitiseert en een staat van hypocorticisme induceert, de HHB as wel zeer responsief blijft voor milde stressoren. De pups zijn reeds op dag drie in staat onderscheid te maken tussen de ervaring dat moeder weg is en de ervaring van een nieuwe omgeving. De eerder genoemde SHRP is dan ook permanent verstoord na herhaalde afwezigheid van de moeder, ook al reageert de HHB as niet meer op haar tijdelijke afwezigheid.

In ***Hoofdstuk 3*** is verder ingegaan op deze resultaten en werd onderzocht of de neuroendocriene desensitisatie op maternale separaties, zoals die gezien en beschreven zijn in het vorige hoofdstuk, komt door een verhoogde glucocorticoïdterugkoppeling. Hiervoor werden de effecten van een MR en GR antagonist gemeten op corticosteronniveaus in bloedplasma om vast te stellen of de HHB as niet meer reageert na herhaalde afwezigheid van de moeder. Wanneer de GR antagonist mifepristone toegediend werd op postnatale dag 5 werd de corticosteronrespons op maternale separatie in de pups versterkt. De GR antagonist had dit effect echter niet wanneer de pups aan herhaalde maternale separaties in de dagen ervoor waren blootgesteld. Onder basale condities tijdens de SHRP (op postnatale dag 5 en 8) zorgde de toediening van de GR antagonist over het algemeen voor een lichte daling in plaats van een stijging in corticosteronniveaus. Maar

na een kleine HHB as activatie, bereikt door het induceren van een milde immuunrespons, werd de GR antagonist wel weer effectief in het verstoren van de GR terugkoppeling en veroorzaakte dan wel een flinke corticosteronverhoging. De MR antagonist spironolactone veroorzaakte een kleine, maar significante verhoging van corticosteron onder basale condities, 8 en 24 uur na toediening. Maar ook na de derde maternale separatie van 8 uur werd een kleine verhoging van corticosteron gezien. De MR antagonist verlaagde juist de corticosteronniveaus, wanneer de pups voor de eerste keer 8 uur werden gescheiden van hun moeder. Tijdens een novelty stressor zorgde een korte blootstelling aan antagonist voor een sterk verhoogde corticosteronrespons, zowel in dieren die een eerste of derde maternale separatie meemaakten. De GR antagonist was ineffectief onder die omstandigheden.

Concluderend ligt een verhoogde glucocorticoïdterugkoppeling dus niet ten grondslag aan de desensitisatie van de HHB as respons op herhaalde maternale separaties en ondersteunen deze resultaten een rol voor de GR in het handhaven van de SHRP. Uit de resultaten blijkt ook de aanwezigheid van een MR-responsief mechanisme, dat de HHB as in toom houdt onder basale, stressvolle en maternale separatie condities tijdens de SHRP. De huidige uitkomsten impliceren dus een MR-responsieve centrale PVN input die de hypofyse-bijnier respons op herhaalde maternale separaties beëindigd, maar die tegelijkertijd de neuroendocriene responsiviteit voor een novelty stressor in stand houdt.

In het tweede deel van dit proefschrift werden de lange termijn effecten van een enkele maternale deprivatie van 24 uur onderzocht. In ***Hoofdstuk 4*** is beschreven dat 6 maanden oude CD1 muizen, die als pup blootgesteld waren aan maternale deprivatie op postnatale dag 8, veranderde cognitieve prestaties laten zien in een ruimtelijke leertaak, de zogenaamde water maze. Dit is een waterbad met een net onder het wateroppervlak verborgen platform als ontsnappingsmogelijkheid. Eén enkele maternale deprivatie zorgde ervoor, dat deze muizen minder goed met een verplaatsing van het platform naar een andere positie in het waterbad konden omgaan (reversal learning) dan muizen uit de controlegroep. Dit geeft aan, dat deze muizen door de maternale deprivatie minder flexibel gedrag vertonen in een verander(en)de omgeving. Echter, het aanleren van een platformpositie en het onthouden van deze positie (lange termijn geheugen) waren niet aangetast. Ook bleef de HHB respons op een novelty stressor onveranderd. Dit impliceert, dat de lange termijn effecten van een enkele maternale deprivatie in muizen zeer subtiel zijn. Deze gegevens ondersteunen de hypothese dat, zoals dat bij ratten het geval is, de lange termijn effecten afhangen van de leeftijd, dus het moment waarop de maternale deprivatie plaatsvindt, en van de leeftijd waarop de effecten onderzocht worden.

In het onderzoek beschreven in ***Hoofdstuk 5*** werden de effecten van maternale deprivatie op verschillende dagen tijdens de SHRP in vroegvolwassen muizen, 'pubers' op postnatale dag 28, vergeleken om meer inzicht te krijgen in de lange termijn effecten beschreven in ***Hoofdstuk 4***. Maternale deprivatie op verschillende dagen veroorzaakte inderdaad lange termijn effecten op markers van de HHB as, zowel onder basale condities als in reactie op milde stressoren, maar

alleen wanneer de deprivatie plaats had gevonden tijdens de SHRP. Maternale deprivatie vroeg in de SHRP (postnatale dag 3) zorgde op postnatale dag 28 voor een verlengde corticosteronrespons op stress en een verminderde basale GR mRNA expressie in het CA1 gebied van de hippocampus, terwijl op een latere leeftijd (postnatale dag 8) maternale deprivatie alleen maar een verhoogde ACTH-respons tot gevolg had. Opmerkelijk was, dat een extra deprivatieperiode (postnatale dagen 3 en 8) de effecten van de eerste deprivatieperiode (postnatale dag 3) beïnvloedde. Onder deze condities werden hoge ACTH concentraties gemeten, maar geen afwijkende corticosteronrespons. Deze resultaten geven nogmaals het belang aan van de leeftijd, en dus van het ontwikkelingsstadium van de hersenen en van de HHB as, waarop de deprivatie plaatsvindt. Het gevolg van een maternale deprivatie hangt dus af van de leeftijd van de pup, en ook van de aard, duur en frequentie van de stressvolle ervaring vroeg in het leven.

De HHB ontwikkeling gedurende de SHRP na maternale deprivatie op postnatale dag 3 is beschreven in ***Hoofdstuk 6*** met als hypothese dat deze ontwikkeling van postnatale dag 4 tot en met 13 veranderd zou zijn. De directe effecten waren deels vergelijkbaar met de veranderingen die beschreven zijn voor maternale deprivatie op postnatale dag 8; dit houdt een verhoogde basale en stressgeïnduceerde ACTH- en corticosteronrespons in, evenals een vermindering van CRH en GR mRNA in de PVN. Er werden nu echter geen directe effecten op MR (in alle subvelden) en GR mRNA (CA1 subveld) expressie in de hippocampus gezien. Wanneer echter op de opeenvolgende dagen gemeten werd, dan bleek de toename van GR mRNA, die normaal gezien wordt tijdens de ontwikkeling, vertraagd te zijn. Eén dag nadat de pups weer herenigd waren met hun moeder leek de SHRP weer (opnieuw) ingesteld te zijn. Blootstelling aan een novelty stressor kon de HHB as in gedepriveerde pups dan niet meer activeren. Bovendien werd een onderdrukking van basaal ACTH en corticosteron gezien voor de drie dagen na de hereniging met de moeder tot niveaus lager dan die gemeten in controle dieren. Maternale deprivatie had geen invloed op de duur van de SHRP, die in beide gevallen na ongeveer 12 dagen ten einde was. Bovendien stelden de directe effecten op centrale HHB markers zich weer geleidelijk in. De terugkeer van de markers naar controleniveaus was echter verschillend voor de markers onderling. GR mRNA expressie in de PVN was slechts één dag verminderd. Dit in tegenstelling tot CRH mRNA expressie in de PVN. Deze was twee dagen verminderd, vervolgens verhoogd op postnatale dag 7, waarna de niveaus langzaam weer afnamen tot het controleniveau. Concluderend heeft maternale deprivatie vroeg in de SHRP dus geen invloed op de duur van de SHRP en zijn de deprivatie-effecten op de HHB as markers tijdelijk van aard.

In ***Hoofdstuk 7*** worden alle resultaten in een breder perspectief besproken. In ***Figuur 7.1*** zijn schematisch de HHB veranderingen weergegeven in de pasgeboren muis na een éénmalige separatie of na herhaalde 8 uur separaties van de moeder. De pups kunnen zich snel aanpassen aan de afwezigheid van de moeder. Dit kan zijn omdat ze na één ervaring haar terugkeer al kunnen voorspellen, maar intussen blijft het HHB systeem wel op een novelty stressor reageren. Voorts geeft een enkele episode van 24 uur maternale deprivatie een aanzienlijke variatie in zowel

de onmiddellijke als de lange termijn effecten. Deze effecten blijken afhankelijk te zijn van de omgeving, de ontwikkelingsfase van het brein, de genetische achtergrond en tenslotte de leeftijd waarop het individu getest wordt.

Conclusies:
De HHB as van een CD1 muizenpup past zich snel aan aan herhaalde dagelijkse maternale separaties van 8 uur, maar blijft wel zeer responsief op blootstelling aan een nieuwe omgeving reageren. De snelle aanpassing van de pup is niet het gevolg van een aangepaste energiehuishouding door het ontbreken van voedsel of door een verhoogde negatieve terugkoppeling, maar lijkt eerder verband te houden met cognitieve processen, die de pup op deze jonge leeftijd al in staat stellen te voorspellen dat de moeder toch wel weer terugkomt. Deze observaties geven dus goed de indrukwekkende plasticiteit van de ontwikkelende hersenen weer.

Na een enkele maternale deprivatie van 24 uur op postnatale dag 3 zijn de HHB parameters slechts tijdelijke verstoord. Op de langere termijn worden echter wel subtiele veranderingen waargenomen in cognitieve taken en HHB activiteit, die afhankelijk zijn van de leeftijd en de genetische achtergrond van de pup, alsmede van de duur en frequentie van de vroege levenservaring.

List of abbreviations

11ß-HSD	11ß-hydroxysteroid dehydrogenase
$5HT_{1A}$	5-hydroxytryptamine (serotonin) 1A
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AVP	vasopressin
BNST	bed nucleus of the stria terminalis
CA	cornu ammonis (part of the hippocampal formation)
CeA	central amygdala
CON	control (mice not separated from their mother)
CRH	corticotropin-releasing hormone
CRHr1	corticotropin-releasing hormone receptor 1
CRP	C-reactive protein
DEP	deprived (pups separated from their mother for 24 hours)
DG	dentate gyrus (part of the hippocampal formation)
GR	glucocorticoid receptor
HPA axis	hypothalamic-pituitary-adrenal axis
IL	interleukin
LG-ABN	licking and grooming - arched back nursing (posture description of the dam for maternal behaviour towards pups)
MC2	melanocortin receptor 2
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NPY	neuropeptide Y
NSEP	non-separated (undisturbed control mice for SEP treated animals)
O.D.	optical densitiy (quantification of mRNA expression)
PEG	polyethylene glycol
pnd	postnatal day
POMC	pro-opiomelanocortin
pPVTh	praventricular thalamic nucleus
PVN	paraventricular nucleus of the hypothalamus
RIA	radio immunoassay
S.E.M.	standard error of the mean
SEP	separated (pups separated from their mother for 8 hours)
SEP+N	separation procedure combined with novelty exposure
SHRP	stress hypo-responsive period

Glossary

adaptation

Adaptation refers to changes in physiological processes and behavioural performance aimed to restore homeostasis.

desensitisation

The reduction of a biological response to repeated exposures to a substance or stimulus.

habituation

Habituation is an example of non-associative learning, in which there is a progressive diminution of behavioural or physiological response, probability due to repetition of a stimulus. If a sensory stimulus is neither rewarding nor harmful the animal learns (consciously or unconsciously) to suppress its response through repeated encounters. Habituation is stimulus-specific.

Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is a complex set of direct influences and feedback interactions between the hypothalamus, pituitary and adrenal that are mediated by hormones, *i.e.* CRH and vasopressin released from the hypothalamic paraventricular nucleus (PVN), pro-opiomelanocortin peptides ACTH and ß-endorphin released from pituitary corticotrophs, and corticosterone (rodent, man) and cortisol (man) secreted from the zona fasciculate of the adrenal cortex. The fine, homeostatic interactions between these three organs control reactions to stressors and regulate various body processes, including behavioural adaptation, mood, energy metabolism, immune and inflammatory responses. See also ***section 1.1.1***.

maternal deprivation

A rodent model to mimic and study the effects of adverse early life experiences on development. In this thesis, this model consists of the separation of CD1 mouse pups from their mother for a single episode of 24 hours. See also ***sections 1.4.5*** and ***1.4.6***.

novelty

An experimental procedure, in which an animal is solitarily placed in a new environment. Usually applied to evoke and study glucocorticoid or behavioural stress responses.

repeated maternal separations

A rodent model to mimic and study the consequences of adverse early life experience on development. In the literature usually daily separations of 3-6 hours from birth to weaning are described. In this thesis, this model consists of up to three daily periods of 8 hours of maternal absence at postnatal days 3, 4 and 5. See also ***sections 1.4.3*** and ***1.4.4***.

sensitisation

Sensitisation is an example of non-associative learning, in which the progressive amplification of a response follows repeated administrations of a stimulus.

stress

"A real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioural responses. In biomedical terms, stress often refers to situations in which adrenal glucocorticoids and catecholamines are elevated because of an experience." As defined by BS McEwen (2000) *Brain Research* 886(1-2): 172-189.

stress hypo-responsive period (SHRP)

A critical period during early development in rodents (rats postnatal days 4 to 14, mice postnatal days 1 to 12) for maturation of the HPA axis. This period is characterised by very low and stable circulating basal levels of ACTH and corticosterone. The responsiveness of the pituitary and adrenal to most mild stimuli is strongly reduced. See also ***section 1.3.1***.

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Curriculum Vitae

Leo Enthoven werd geboren op 16 oktober 1976 in de gemeente Amsterdam. Hij haalde zijn atheneum diploma in 1995 aan het Zwijsen College te Veghel. In september van datzelfde jaar begon hij zijn studie biologie aan de Universiteit Leiden, waar hij in 2000 afstudeerde. Tijdens deze studie heeft hij verschillende onderzoeksstages gedaan: een hoofdvakstage van 9 maanden bij Medische Farmacologie (LACDR, LUMC, Universiteit Leiden) onder begeleiding van Dr. M.S. Oitzl en Prof. Dr. E.R. de Kloet, een buitenlandstage van 5 maanden bij Neurobiologie aan het Deutsches Primaten Zentrum in Göttingen, Duitsland onder begeleiding van Dr. E. Isovich en Prof. Dr. E. Fuchs en een nevenstage van 6 maanden bij Evolutiebiologie (EEW, Universiteit Leiden) onder begeleiding van Dr. A. Monteiro en Prof. Dr. P. Brakefield. Aansluitend was hij werkzaam als promovendus bij Medische Farmacologie, onderdeel van het Leiden/Amsterdam Center for Drug Research en het Leids Universitair Medisch Centrum, onder begeleiding van Prof. Dr. E.R. de Kloet en Dr. M.S. Oitzl. Het daar uitgevoerde onderzoek staat beschreven in dit proefschrift. Dit onderzoek was onderdeel van het NDRF-project "Nuclear receptors: novel targets for tissue-specific antidepressants", een samenwerking tussen de Universiteiten van Groningen, Amsterdam en Leiden, het Huybrecht Laboratorium (Utrecht) en Organon N.V. (Oss). Sinds januari 2007 werkt hij als statistisch onderzoeker in de sector Indexcijfers prijzen en conjunctuur, onderdeel van de Divisie Macro-economische Statistieken en Publicaties van het Centraal Bureau voor de Statistiek.

Leo Enthoven was born on October 16th, 1976 in Amsterdam. In 1995 he graduated from high school (atheneum) at Zwijsen College in Veghel. Later that year he started his Biology degree at Leiden University, which he completed in 2000. During his study he performed several internships: a main internship of 9 months at Medical Pharmacology (LACDR, LUMC, Leiden University) under supervision of Dr. M.S. Oitzl and Prof. E.R. de Kloet, an internship of 5 months at Neurobiology at the Deutsches Primaten Zentrum in Göttingen, Germany under supervision of Dr. E. Isovich and Prof. Dr. E. Fuchs, and an internship of 6 months at Evolutionary Biology (EEW, Leiden University) under supervision of Dr. A. Monteiro and Prof. Dr. P. Brakefield. Subsequently, he performed his PhD research at Medical Pharmacology, part of the Leiden/Amsterdam Center for Drug Research and Leiden University Medical Center under the supervision of Prof. Dr. E.R. de Kloet and Dr. M.S. Oitzl. The work from this period is presented in this thesis. This research was part of the NDRF-project "Nuclear receptors: novel targets for tissue-specific antidepressants", a collaboration between the Universities of Groningen, Amsterdam and Leiden, the Huybrecht Laboratory (Utrecht) and Organon N.V. (Oss). Since January 2007 he works as a statistical researcher in the department of Price statistics and short-term indicators, part of the division Macro-economic Statistics and Dissemination of Statistics Netherlands.


Bijzonder project, veel geleerd
Eindelijk klaar, in boekvorm gepresenteerd
Dagen vol experimenten, zelden alleen gezwoegd
Altijd een helpende hand, dat deed me goed
Naast collegae ook vrienden, voor afleiding en plezier
Kan ik nu met trots zeggen: als Dr. sta ik hier
Tis af!

Leo

Plasma corticosterone responses reflect the degree of novelty in male and female CD1 mice

Appendix I

L. Enthoven, S. Dalm, E.R. de Kloet & M.S. Oitzl

The studies described in this appendix provide methodological considerations for blood sampling via tail incision in mice, a technique that was also used for the determination of the corticosterone responses to swim stress in maternally deprived and control mice described in ***Chapter 4***.

I.1 Abstract

The hypothalamic-pituitary-adrenal axis response to novelty stress is dependent on the type and duration of a certain challenge. Here, we show in CD1 mice that corticosterone secretion increased reflecting the "degree of novelty" (home cage < 5 minutes in cylinder < 60 minutes in cylinder < 1 minute forced swim). The magnitude of the corticosterone response already differed 15 minutes after onset of the stressor, with the more severe challenge causing a faster initiation of the glucocorticoid-related stress response. Furthermore, female mice secreted more corticosterone than male mice under basal conditions and showed a faster and higher increase in response to each novelty.

I.2 Introduction

A variety of physiological [1, 11, 13] and emotional [9, 10, 12, 15] stimuli result in different behavioural coping responses [1, 16, 21]. Any challenge, either real or imagined, evokes a glucocorticoid-related stress response, which serves to restore homeostasis and facilitate behavioural adaptation [17]. Some challenges, however, elicit stronger corticosterone responses than others [6, 10, 12, 13]. In our lab, we generally place rats or mice in a novel environment and estimate plasma corticosterone concentrations at several time points during this novelty exposure [7, 8, 18, 24, 25]. The aim of the present study was to define novelty conditions characterised by differential corticosterone secretion. In future experiments, these should allow to choose the challenge according to the hypothesised change in corticosterone secretion, thus avoiding ceiling effects.

Here, we varied both the kind and duration of novelty. Male and female CD1 mice were placed into a cylinder for 5 or 60 minutes, swam in a large pool with warm water for 1 minute or remained in their home cage. Blood samples were taken sequentially 15, 30 and 60 minutes after onset of novelty, *i.e.* in the proactive phase of the glucocorticoid-related stress response [2, 3]. Depending on the challenge we expected a different amplitude or slope of the corticosterone response and overall higher corticosterone concentrations in female than in male mice.

I.3 Materials and Methods

I.3.1 Animals

Male and female CD1 mice (6 months of age) were housed four per cage in same sex groups with food and water *ad libitum* under a fixed 12 hours light/dark cycle (lights on at 07:00 hours) at

Table I.1: Overview of the challenges with their respective sampling time points

		sample time point (minutes)				n =	
		0	15	30	60	male	female
Basal:		x				20	20
Novel environment:	5'CYL	-	x	x	x	16	16
	60'CYL	-	x	x	x	16	16
	SWIM	-	x	x	x	20	20
Control:	RSC	-	x	x	x	16	16
	RHC	-	-	-	x		13

In this experiment animals were divided over different treatment groups: *Basal*; *Novel environment:* 5'CYL = 5 minutes exposure to a cylinder, thereafter replacement in home cage; 60'CYL = 60 minutes exposure to a cylinder; SWIM = 1 minute forced swimming, thereafter placement under a heating light for 3 minutes and replacement in home cage; *Control:* RSC = repeated sampling control; RHC = repeated handling control. All treatments started at t=0 minutes and all handling procedures, with the exception of actual blood sampling, were equal in groups 5'CYL, 60'CYL, SWIM, RSC and RHC. (" x " indicates a blood sample taken, " - " indicates no blood sample taken.)

the animal facilities of the Sylvius Laboratory at Leiden University, The Netherlands. Testing was between 09:00-13:00 hours. Mice were singly housed one week before the experiment started. Experiments were approved by the Local Committee of Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

I.3.2 Evaluation of novelty intensity

The treatment groups are summarised in ***Table I.1***. *Basal:* To determine basal morning corticosterone concentrations, blood samples were collected from male and female mice within two hours after lights on (n=20 per sex). *Novel environment:* Mice (n=16 per sex and condition) were placed in an upright cylinder (gray PVC; 17 cm high, 9.5 cm in diameter) for 5 minutes before they were returned to their home cage or for 60 minutes (groups 5'CYL and 60'CYL, respectively). Another group of mice (n=20 per sex) was placed in the middle of a pool with warm water (140 cm in diameter; 26±1°C) and had to swim for 1 minute (group SWIM). Thereafter, the mouse was placed in a clean cage with tissue bedding under a heating lamp for 3 minutes and returned to its home cage. In all conditions, of each mouse three sequential blood samples were taken 15, 30 and 60 minutes after the start of the challenge. After each blood sample, mice returned to their home cage (5'CYL, SWIM) or cylinder (60'CYL). *Control:* To control for the effects of repeated blood sampling we used two home cage control groups. Repeated sampling control mice (group RSC, n=16 per sex) were removed shortly from their home cage, sequential blood samples were taken at t=15, 30 and 60 minutes, and after sampling mice returned to their home cage to mimic the handling effects involved for the introduction to a novel environment. In the repeated home cage control group (group RHC, n=13 females), we performed the same handling procedures, but without actual tail incisions at t=15 and 30 minutes. Only at t=60 minutes a blood sample was collected.

I.3.3. Blood collection and corticosterone determination

Blood samples were collected by tail incision, a method that allows estimation of basal concentrations of corticosterone and multiple samples from the same animal [4, 5]. Briefly, the mouse is placed on the grid top of its home cage. A small incision at the base of the tail with a razor blade allows collection of 50-100 µl blood within 10-15 seconds, without anesthesia.

Blood was collected in 100 µl capillaries, coated with potassium-EDTA, and centrifuged for 10 minutes at 13000 rpm at 4°C. Plasma was separated from the pellet and stored at -20°C. Corticosterone was measured with a ^{125}I-corticosterone radio immunoassay (RIA) kit (ICN Pharmaceuticals Inc., New York, U.S.A.).

I.3.4 Data analysis

Data are presented as mean ± S.E.M. Plasma corticosterone concentrations were analysed by ANOVA (factor: sex=2) when appropriate with repeated measures (at 15, 30 and 60 minutes; factors: SEX=2; CONDITION=4: groups RSC, 5'CYL, 60'CYL, SWIM) and by LSD *post hoc* test. Statistical significance was accepted at $P<0.05$.

I.4 Results

Basal corticosterone concentrations were low in both male and female CD1 mice, and as expected significantly higher in female than in male mice ($F(1,38)17.10$, $P<0.001$: male 12.2 ± 1.7 ng/ml; female 33.4 ± 4.8 ng/ml).

Male mice secreted less corticosterone than female mice in response to each challenge ($F(1,128)185.06$, $P<0.001$; ***Figure I.1***). The magnitude of the corticosterone secretion depended on the challenge ($F(3,128)10.29$, $P<0.001$). Corticosterone concentrations increased with time ($F(2,256)274.58$, $P<0.001$), showed a different course in male and female mice (interaction time x sex: $F(2,256)11.45$, $P<0.001$), depending on the experimental condition (interaction time x sex x condition: $F(6,256)3.79$, $P=0.001$). The highest corticosterone concentration was observed for the SWIM condition and the lowest concentration for the home cage control (group RSC).

I.4.1 Male mice

At $t=15$ minutes, corticosterone secretion was significantly increased in relation to the degree of novelty: lowest in the home cage control (RSC: 30.8 ± 7.5 ng/ml; vs. 5'CYL, 60'CYL or SWIM $P<0.001$), followed by mice exposed for 5 minutes to the cylinder (5'CYL: 61.2 ± 6.7; vs. 60'CYL or SWIM $P<0.05$) and mice continuously exposed to the cylinder (60'CYL: 121.2 ± 15.0 ng/ml), with the latter similar to the SWIM group (106.4 ± 17.5 ng/ml). At $t=30$ minutes, corticosterone concentrations were further increased: equally high in groups RSC and 5'CYL, and both significantly lower than 60'CYL and SWIM ($P<0.01$). At $t=60$ minutes, corticosterone concentrations were again further increased and differed between all groups ($P<0.001$ to $P<0.05$), with highest concentrations in the SWIM group.

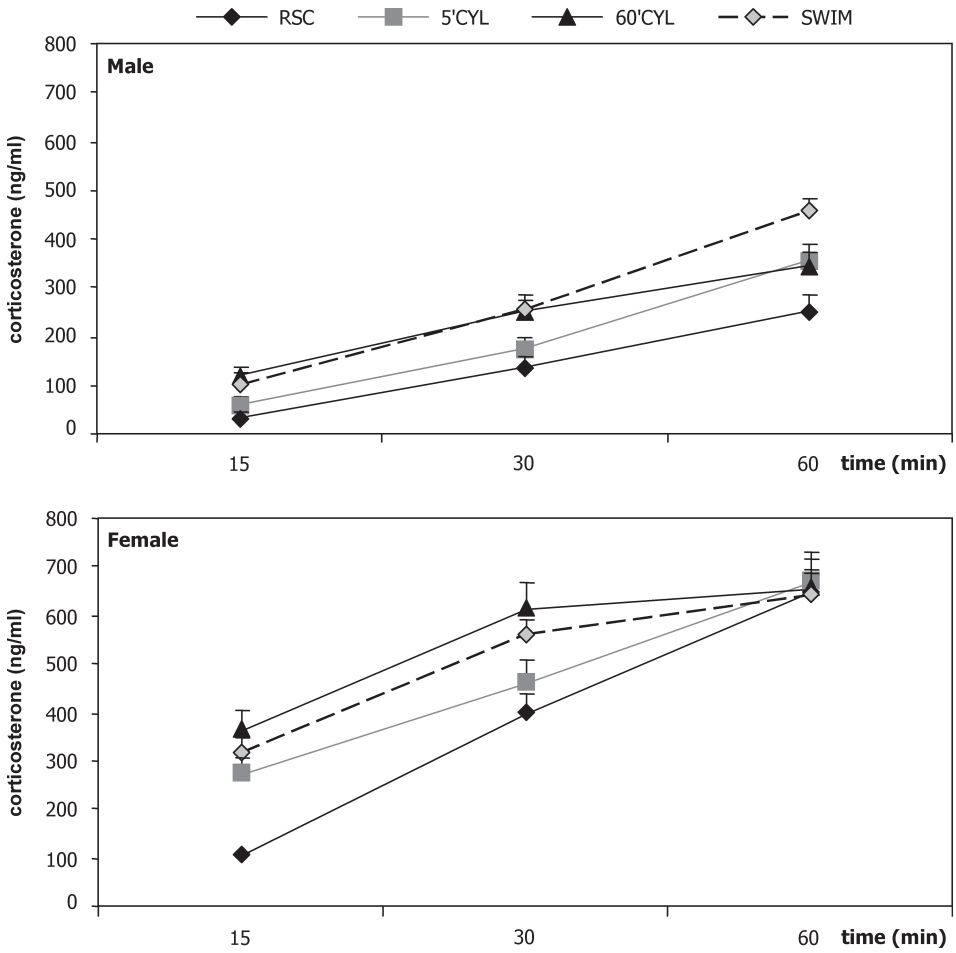

Figure I.1
Novelty-induced corticosterone response for male and female CD1 mice, measured 15, 30 and 60 minutes after initiation of each stressor. For abbreviations see ***Table I.1***. Values are presented as mean ± S.E.M.

I.4.2 Female mice

At *t*=15 minutes, corticosterone was significantly lower in group RSC (101.1 ± 9.2 ng/ml; *P*<0.001) than in groups 5'CYL (272.6 ± 35.1 ng/ml), 60'CYL (364.1 ± 41.7 ng/ml) and SWIM (313.2 ± 33.8 ng/ml). At *t*=30 minutes, corticosterone further increased, but was still lowest in RSC (*P*<0.01) compared to 60'CYL and SWIM. At *t*=15 and 30 minutes, corticosterone of group 60'CYL was significantly higher than of group 5'CYL (*P*<0.05), but similar to group SWIM. At *t*=60 minutes, corticosterone was further increased, but showed the same concentrations in all four groups.

Mimicking the blood sampling procedure without actual tail incision at *t*=15 and 30 minutes, corticosterone concentrations measured in female mice RHC group at *t*=60 minutes (35.5 ± 6.6 ng/ml) were similar to basal conditions.

I.5 Discussion

We differentiated between the "degree of novelty" of a challenge and gender by analysing the plasma corticosterone concentrations in the proactive phase of the glucocorticoid-related stress response [2, 3]. The duration of exposure to the same challenge (*i.e.* 5'CYL versus 60'CYL) affected

the slope of the stress response as corticosterone secretion was initiated faster. This initial faster secretion of corticosterone resulted in more secreted corticosterone after 60 minutes, though ceiling effects were already observed at 60 minutes. One minute forced swimming (group SWIM), followed by three minutes under a heating lamp before returning to the home cage resulted in a similar response as group 60'CYL, but with a higher amplitude in male mice.

Besides challenge effects, female mice also responded differently than males to the challenges. Female mice showed higher corticosterone levels, basal and in response to each of the challenges. Furthermore, female mice seem to reach a plateau of corticosterone secretion where males do not. These observations are consistent with literature, showing that estrogens increase corticosteroid-binding globulin values resulting in higher total plasma corticosterone concentrations [3, 14, 19]. Irrespective of gender, the main factor of group differences indicating the degree of novelty (RSC < 5'CYL < 60'CYL < SWIM) was determined by the shape of the stress response in the proactive phase.

Marquez *et al.* [13] showed in rats that the recovery speed of the stress system (*i.e.* reactive phase) differs after administration of either foot shock or immobilisation. They too found a maximal corticosterone response 60 minutes after initiation of both challenges, but were unable to distinguish between the two stressors in the proactive phase. Combined with our observations these experiments indicate that, despite ceiling effects, different stimuli elicit different proactive and reactive stress responses. Furthermore, we suggest that both proactive and reactive aspects of the stress response may reflect the intensity of a given challenge [6, 10, 12, 13].

It was striking and unexpected to notice that even the repeatedly sampled control group (group RSC) elicited a stress response, though animals returned to their home cages. Rats responded differently to repeated sampling via tail incision, as was shown by Fluttert *et al.* [5]. In their study frequent sampling of 300 µl of blood from handled Wistar rats with an interval of 20-30 minutes only slightly elevated corticosterone levels. Blood samplig via tail incision was therefore considered not to be harmful to the animal [4, 5]. It is possible that the response we observed was not due to the procedure of tail incision itself, but to the frequency of blood sampling. Mice have a much smaller blood volume than rats and we estimated that our mice lost up to 20 % of their total blood volume after three consecutive samples within a time span of 60 minutes. Activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to occur at approximately 12% plasma volume deficit [20, 22, 23]. To assess this possibility a naive group of female mice received the same handling procedures as group RSC, including a virtual sampling using the blunt side of the razor blade, but without actual incision and blood loss at 15 and 30 minutes (group RHC; we expect the same results in males). Only at 60 minutes a blood sample was taken. These handling procedures by itself did not affect circulating corticosterone concentrations. Consequently, it is most likely that dehydration might explain the rise in corticosterone observed in group RSC. Each of the challenges still elicited a faster and higher corticosterone response than group RSC, therefore effects of different challenges on the HPA axis responsiveness were still detectable using repeated sampling. However, considerations have to be made when designing an experiment on

the number of consecutive tail samples and the time frame in which these samples are taken.

In conclusion, this survey demonstrates that different challenges or varying exposure times to the same challenge causes different reaction patterns in both male and female CD1 mice. These effects were observed in the proactive phase of the corticosterone stress response and indicate plasticity for coping with a novel environment.

I.6 Acknowledgements

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