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Early life experience : neuroendocrine adaptations to maternal absence

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Introduction

Chapter 1

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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]

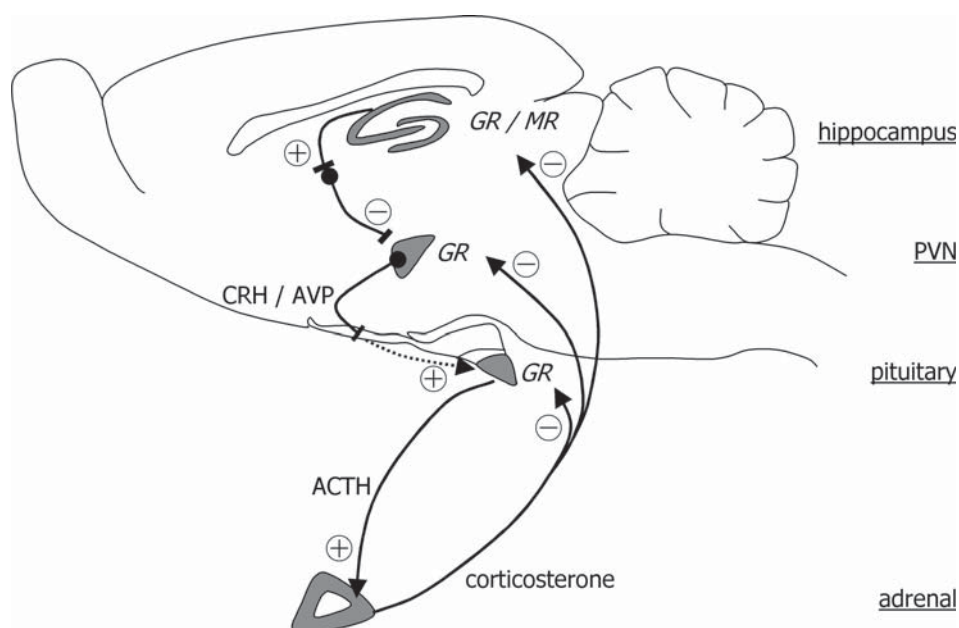


Figure 1.1

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 paraventricular nucleus (PVN) of the hypothalamus from brain stem 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 adrenocorticotrophic hormone (ACTH) into the blood stream. ACTH stimulates the adrenal cortex to secrete 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 adrenocorticotrophic 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 encounter 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 11 β -hydroxysteroid dehydrogenase (11 β -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 exemplify 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

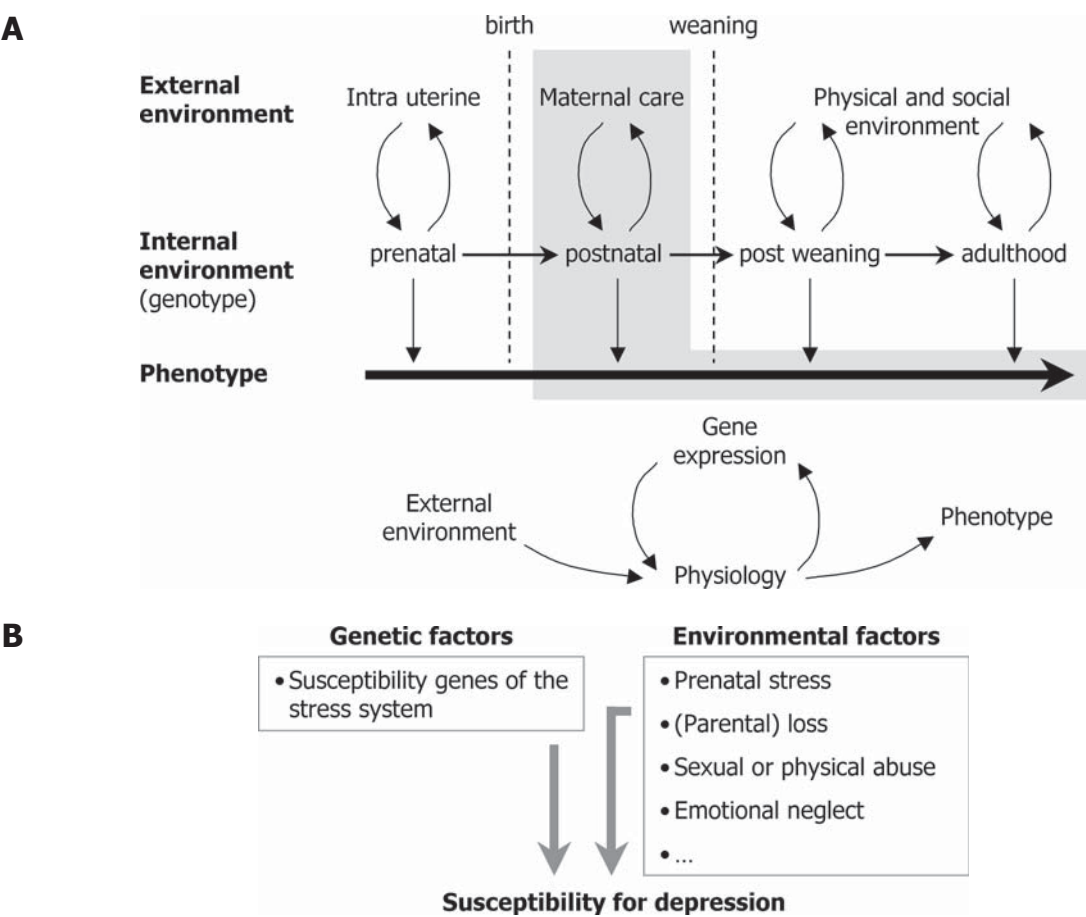


Figure 1.2
(A) A schematic overview of the interactions between the genome and environment shaping the phenotype of an organism throughout its life. The focus of this thesis is on early postnatal period between birth and weaning in mice (gray area). Variations in maternal care or physiological changes 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

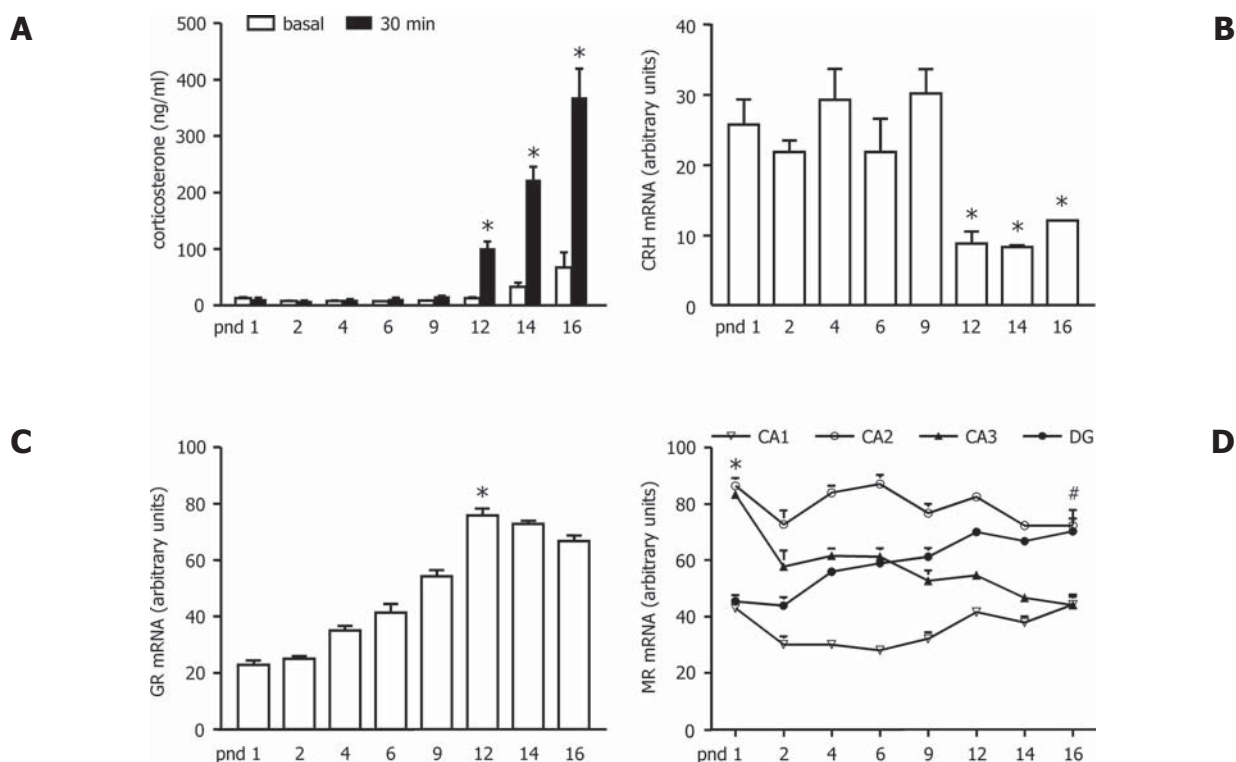


Figure 1.3

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

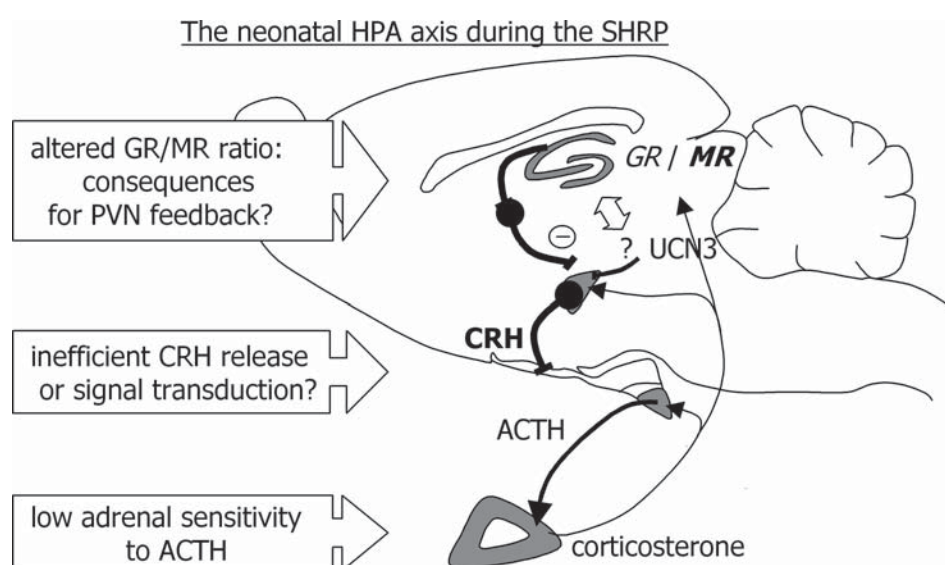


Figure 1.4 Schematic illustration of the stress system during the SHRP indicating the significantly altered state and function of the HPA axis at different organisational levels as compared to the adult. See **Figure 1.1** for abbreviations and an explanation of the adult stress response. Adapted from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

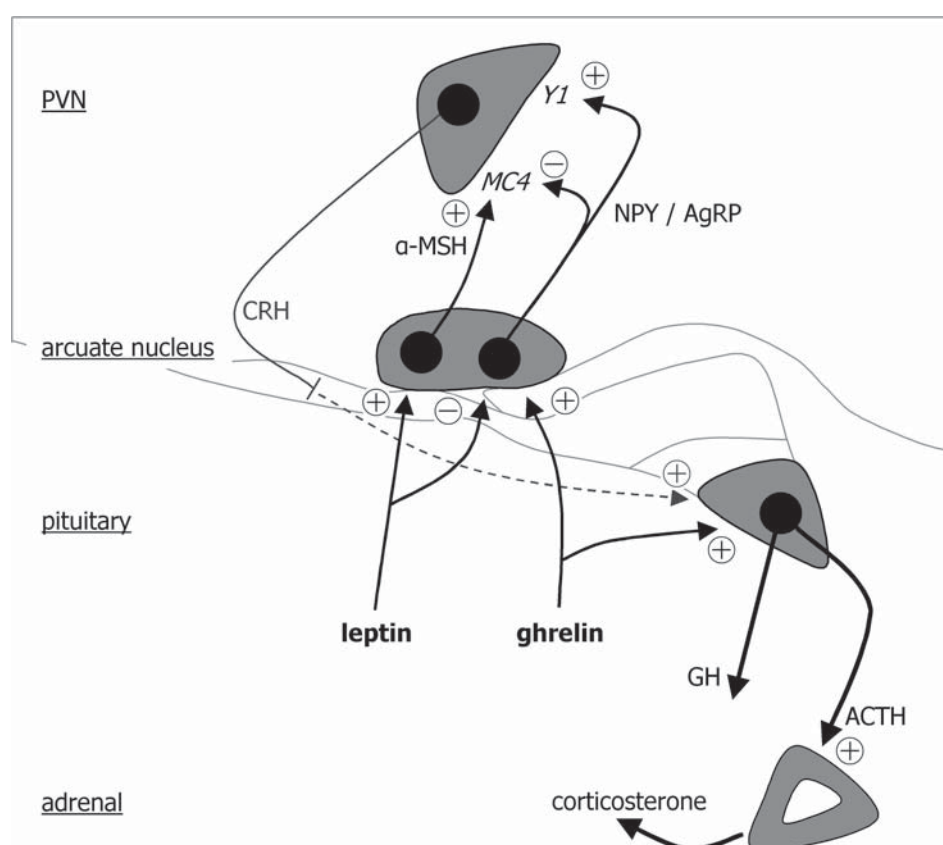


Figure 1.5

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 adrenocorticotrophic 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 accurately 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-

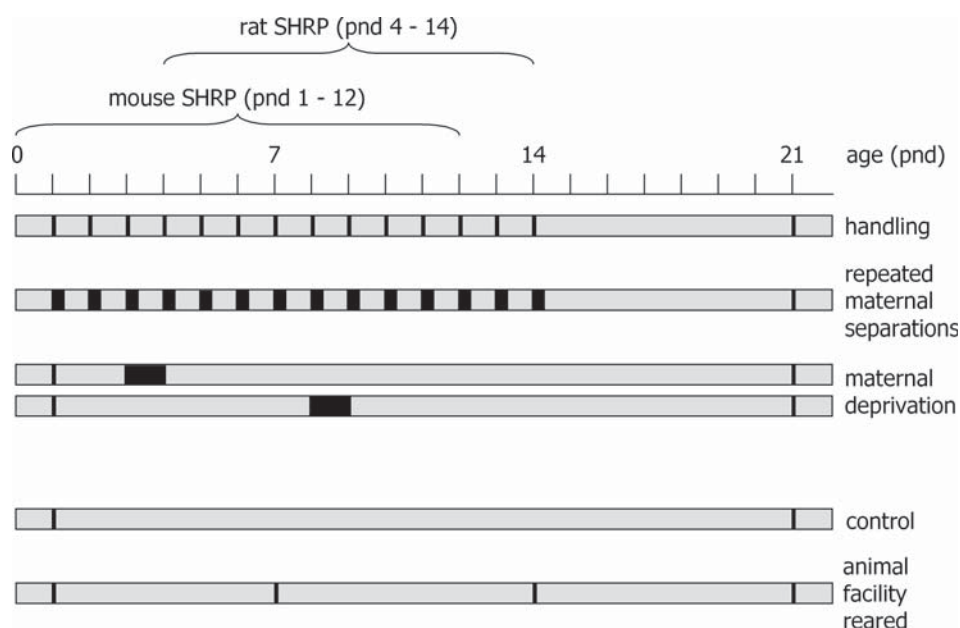


Figure 1.6

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 various 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 infant's 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.

1.6 References

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