Early life experience : neuroendocrine adaptations to maternal absence

Enthoven, L.

Chapter 7

General discussion

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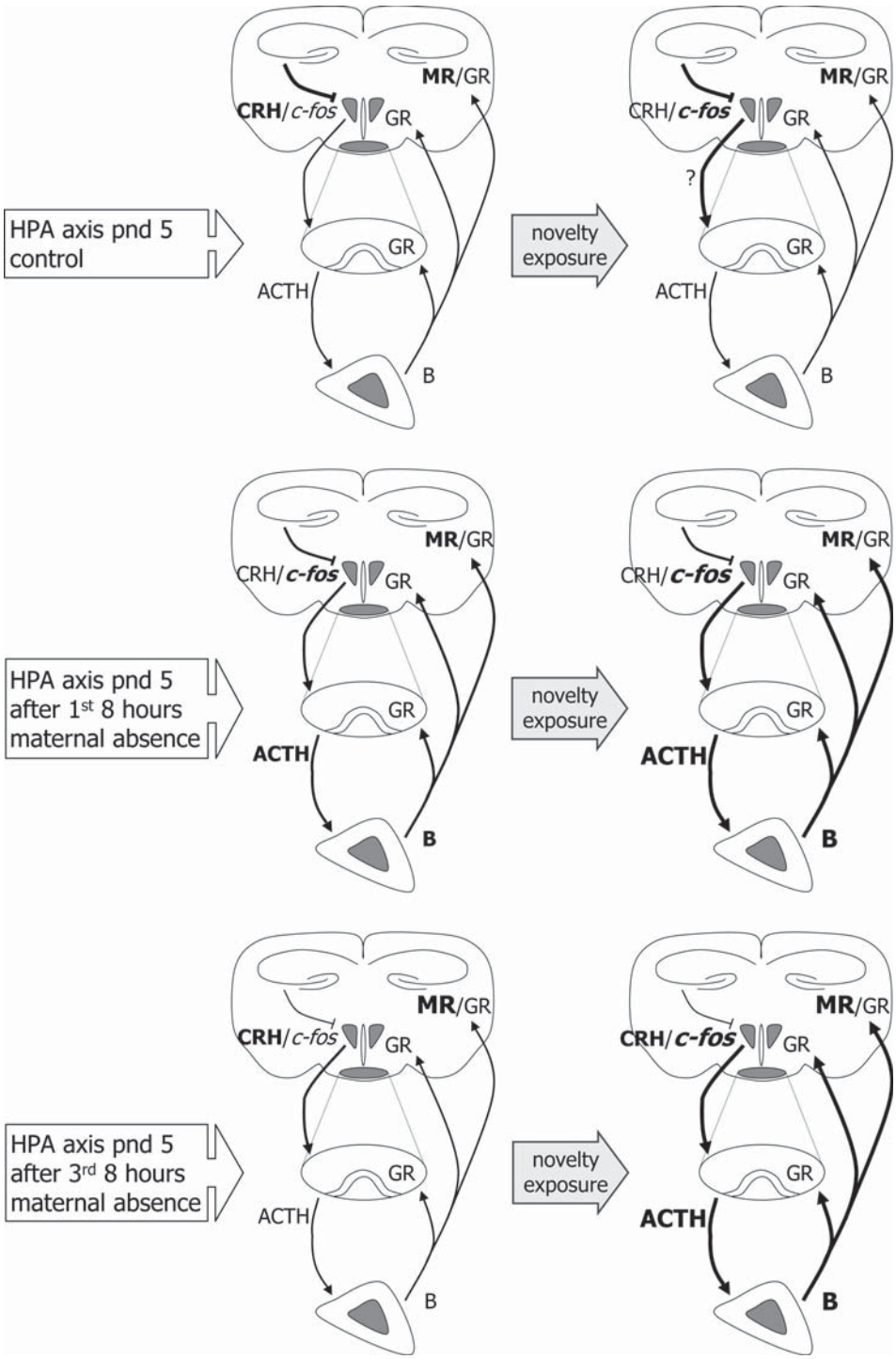
The objectives of the studies presented in this thesis were to examine in mice the immediate and lasting effects of adverse early life experiences on the HPA axis and behaviour. For this purpose two paradigms were used. One paradigm was based on the repeated daily 8 hours separations of CD1 mouse pups 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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