Programming of hippocampal structure and function by early-life stress: Opportunities for nutritional intervention
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Chapter 7

General Discussion

Food for thought
Chapter 7

OUTLINE

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1. INTRODUCTION

Early-life experiences can have a life-long impact on mental health. Adverse early-life experiences (e.g. physical/sexual abuse, neglect, rearing by a drug or alcohol abusive mother, exposure to war, poverty, illness or institutionalization) are associated with an increased risk to develop psychopathologies [1,2] and cognitive impairments in adulthood [3-6]. Although a large body of data firmly establishes this association between early-life stress (ES) and later-life mental health outcomes, the underlying biological mechanisms remain largely elusive. Increasing our insight into the mechanisms implicated in early programming can aid the development of intervention strategies, which is of importance, given that exposure to early-life stress (ES) is incredibly common [7], often difficult to prevent, and currently no intervention strategies are available to protect a vulnerable population against the lasting consequences of ES.

The main aims of this thesis were to i) gain more insight into the biological processes at play during the early sensitive postnatal period(s) in life, when environmental experiences, hormonal influences and nutrition interact and can confer enduring effects on later brain structure and function, and ii) to test the efficacy of interventions that are designed based on this knowledge. We concentrated specifically on the effects of chronic early-life stress (ES) in mice on the hippocampus, a highly plastic brain region, important for cognition and for regulation of the stress response. In the first part of this chapter, we discuss the ES model (section 2) used in this thesis, and address why neurogenesis was studied as a potential neurobiological substrate of ES-induced cognitive impairments (section 3).

Next we discuss three more general issues relevant for the early programming by ES, namely: the complexity of the early-life environment, critical time-windows and sex-specific vulnerability. In order to design interventions, it is crucial to better understand: i) which components of the early-life environment are key in programming ES effects; ii) if the ES effects are progressively developing, or whether they irreversibly impact the brain from the start; and iii) if ES differentially affects males or females. In the second part of this chapter, we take each of these three questions as a starting point to place our findings in a broader perspective and address the possible implications for interventions (sections 4-6). Lastly, we discuss the adaptive significance of early programming for the longer term (section 7).
2. CHRONIC EARLY-LIFE STRESS IN MICE; THE LIMITED NESTING/BEDDING MATERIAL MODEL

In the wild, neonatal mice spend most of their time in a safe and stable nest while the dam protects them and provides them with food and cues about their (future) habitat. During the first days of their life, the presence of the dam thereby represents the primary source of nutrition, warmth and sensory stimulation for the pups. The quality of the early-life environment is therefore largely determined by factors embedded in this dam-pup relationship and can thus also be directly affected by alterations in maternal behavior. Proper maternal care is in turn dependent on characteristics of the dams’ environment (e.g. food availability, predator pressure, or, in the laboratory setting, the availability of sufficient amounts of nesting/bedding material) [8,9]. Maternal care, in mice and rats, is temporally distributed in nursing epochs, during which the dam gathers her pups in the nest and under her body to nurse them, while providing short bouts of grooming and anogenital licking, a specific form of behavior that is necessary to promote urination and defecation by the pups. Frequency, distribution, duration and quality of the nursing epochs are essential to maintain the pup’s bodily homeostasis. Each of these variables can serve as an environmental cue about the pups’ future habitat.

As an experimentally induced disruption of the normal dam-pup relationship is very powerful in inducing ES, the limited nesting/bedding material paradigm was developed [10] and used in this thesis. This model provokes stress in the dams and fragments her maternal care, which subsequently induces chronic ES in her offspring. While this paradigm fragmentizes maternal nest attendance (chapters 2 and 4), it does not reduce the total amount of nursing. In comparison with other postnatal ES models in rodents (e.g. maternal separation and maternal deprivation paradigms), the limited nesting/bedding model induces chronic instead of acute/recurrent stress, and, as the dam remains present, this is delivered in a more naturalistic manner [11]. Thereby, the model recapitulates important aspects of human ES situations in which the stress is typically chronic and often results from abnormal, inconsistent and/or abusive behavior of a present mother [12].

Importantly, the limited nesting model involves minimal interference by the researcher; only on the mornings of P2 and P9 the researcher disturbs the cage. Potential handling effects are thus largely prevented. However, because of this, daily monitoring of individual parameters (e.g. food-intake, maternal care, excretion, hormone levels or vocalization) of individual pups is not possible during the ES period, as this would interfere too much with the model. This could be important as previous studies have reported within-litter
variation in the amount of care that each individual pup receives [13], next to the fact that males seem to receive more maternal care in comparison with their female littermates [14]. Other practical issues that should be considered when using this model, include the prevention of bias introduced by e.g. variation in the prenatal environment and natural variation in maternal care. In the present research, we took care to control for such confounding factors by: i) breeding in house; ii) using only primiparous (inexperienced) females as mothers; iii) limiting litter size to 5-6 pups; iv) observing and quantifying levels of maternal care; v) including animals from multiple litters within one experimental group and statistically controlling for litter effects.

In the controlled setting of the laboratory, the limited nesting model represents a naturalistic stressful early life environment, in all its complexity. In section 4 we address the various components of this complex environment, their interactions and their role in programming brain structure and function in early-life. Despite the obvious limitations of rodents to reproduce the full rich repertoire of human development and cognitive and emotional behaviors, animal models –such as the limited nesting model– provide important tools to study the role of early-life experiences in later resilience and vulnerability to cognitive disorders, and in the underlying neurobiological substrates of such behaviors. In the next section we discuss our focus on the hippocampus and hippocampal neurogenesis.

3. HIPPOCAMPAL NEUROGENESIS

3.1 THE HIPPOCAMPUS, SUSCEPTIBLE TO EARLY-LIFE EXPERIENCES
Amongst the most striking long-term effects of ES exposure in humans are impairments in adult cognitive function [3-5]. These cognitive deficits have been associated with reductions in volume of the hippocampus [15,16], a brain region crucial for the formation and retrieval of memories [17,18]. Interestingly, reduced hippocampal volumes have also been found in adults born with a low birth weight and are very common in ES-associated disorders, including major depression [19,20], especially in patients with a history of childhood adversity [21].

Next to its role in cognition, the hippocampus is involved in the regulation of the stress response and richly endowed with glucocorticoid and mineralocorticoid receptors [22,23]. The fact that hippocampal development is not complete at birth, and that its maturation extends into the postnatal period [24,25] makes this brain region particularly susceptible to disturbances, or stress exposure, during this early postnatal period. Indeed, in various animal studies, the association between ES-induced impairments in hippocampus-dependent cognitive tasks (e.g. deficits in spatial/object learning and memory) and
alterations in hippocampal structure and plasticity has been confirmed. The reported ES-induced alterations range from reduced dendritic complexity, reduced spine density, and altered electrophysiological properties to alterations in adult neurogenesis (AN)(see [26] for an overview).

3.2 Hippocampal neurogenesis as a neurobiological substrate for ES-induced cognitive impairments?

In this thesis, we studied effects of ES on hippocampal adult neurogenesis (AN). Compared to the generation of new spines, synapses and dendrites, the formation of completely new neurons, and their functional integration into an existing adult circuit, can be considered an extremely plastic change that enables the adult brain to adapt to its environment. Hippocampal AN does not just ‘replace’ old neurons, but rather adds new ones to an existing circuit. A full understanding of the functional relevance of hippocampal AN is still developing; some consider AN an evolutionary ‘leftover’, or remnant of delayed postnatal development, whereas others show that the seemingly small fraction of newly generated neurons can contribute significantly to hippocampal functioning.

There is accumulating evidence indicating that increases in AN are associated with enhanced cognitive performance [27-29], while suppression of AN is paralleled by reductions in learning and memory [30-32]. In addition, the newly generated, young neurons are more excitable than mature granule cells [33], located in a strategically important location of the trisynaptic circuit, and are preferentially recruited upon (behavioral) activation of the hippocampus [34]. However, it remains a challenge to causally prove the behavioral relevance of AN per se [35]. Neurogenesis has now been implicated in; pattern separation [28,36], memory processing, memory clearance, reduction of interference and forgetting [37,38]. While small numbers of cells can be functionally implicated in such complex behaviors, evidently, a single behavior never depends exclusively on one single form of neuronal plasticity and other forms of plasticity often contribute as well.

In chapter 2, we focused on the behavioral relevance of (altered) neurogenesis in (ES-exposed) mice. We studied if ES alters AN, and questioned if this could be one of the neurobiological substrates of ES-induced cognitive deficits by testing if AN correlates with, and statistically accounts for, changes in cognitive functions. We showed that ES reduced survival of newborn neurons without altering levels of proliferation and differentiation, consistent with the idea that it is not the absolute levels of cell proliferation (which are largely determined by age), but rather the altered levels of newborn cell survival that might underlie the adaptive response that fine-tunes hippocampal plasticity [39].
We showed that spatial memory in the object location task and in the Morris Water maze probe trial is AN-dependent, establishing covariation between neurogenesis and performance in these behavioral tasks. However, performance in the object recognition task, also impaired by ES, was not correlated with the survival of newborn neurons, indicating that this task is not, or less AN-dependent. Indeed, in chapter 4, after dietary intervention, the lasting reduction in neurogenesis persisted as well as the impaired performance in the AN-dependent object location task, while performance in the AN-independent object recognition task was no longer found to be impaired after ES. This indicated that intact object recognition can occur even with reduced levels of AN. However, others have reported that the object recognition task is AN-dependent [30], albeit in a slightly different behavioral testing paradigm, and when using rats instead of mice. In line with our findings, hippocampal lesion studies have shown that the hippocampus is crucial for recognition when the memory concerns a spatial or temporal component (as in the object-location task) but not for recognizing object familiarity (as in the object recognition task) [40].

Our findings indicate that hippocampus-dependent learning and memory (at least partly) depends on neurogenesis. While neurogenesis is most likely not the only form of hippocampal plasticity that contributes to cognitive function, it remains important, considering that neurogenesis is strongly modulated by life experience and could be used as a good indicator of health status of the brain, particularly in mice, as explained below.

3.3 Why study neurogenesis?

Neurogenesis has been demonstrated in a wide range of species [41], including humans [42-44] and it is abundant in rodents. The fact that neurogenesis is strongly modulated by environmental factors, indicates that it might be relevant for adaptation and for maintaining homeostasis of the brain [45]. Interestingly, various environmental factors that negatively affect neurogenesis (e.g. stress, sleep deprivation and inflammation) have also been implicated in the pathophysiology of mood disorders [46]. In addition, alterations in neurogenesis likely contribute to the hippocampal (cognitive) aspects of psychopathologies like schizophrenia and neurodegenerative disorders [47]. In this thesis we showed that early-life experiences modulate neurogenesis and adult neurogenic capacity. As ES increases susceptibility to several psychopathologies [48,49], it can not be excluded that this is (partially) mediated by altered levels of neurogenesis. In addition, an extra reason to study this particular form of hippocampal plasticity is to learn how neurogenesis is modulated as this might contribute to novel therapeutic strategies for structural brain repair, e.g. to replace neurons lost due to stroke, injury or neuro-degeneration.
It is interesting to consider that neurogenesis might reflect a different relevance for brain function and species-specific behaviors in rodents compared to other mammals. In fact, rodents have a relatively short life (± 2-3 years) and accordingly they can already reproduce relatively early in life (around 3-4 weeks of age), when levels of neurogenesis are still relatively high. This is in contrast with longer living mammalian species, like primates, in which such critical events occur later in life, when the number of proliferating cells in the hippocampus is already drastically diminished [39]. Therefore, high rates of neurogenesis in rodents might possibly be needed for behavioral flexibility required during major life-events associated with adolescence and early adulthood. Interestingly, natural behaviors such as sexual reproduction [50,51] and mother- [52,53] and parenthood [54] indeed modulate neurogenesis in rodents. The fact that neurogenesis seems involved in behaviors critical for the existence of the species suggests that its functional relevance extends beyond the scope of spatial memory alone.

Understanding how ES affects relevant neurobiological processes in the mouse might help us understand the mechanisms via which lasting effects are exerted on brain structure and function. Therefore, we first need to comprehend which components of the early-life environment are critical.

4. THE NEUROBIOLOGY OF EARLY-LIFE STRESS

4.1 BEYOND MATERNAL CARE

Similar to the human situation where the early environment encompasses multiple elements, models disrupting the mouse dam-pup relationship (described in section 2) alter many key components of the early-life environment at once; in these models, no single factor acts alone. This makes it difficult to disentangle the separate contributions of the individual components. The challenge of understanding early-life programming is not to isolate the influence of one component, while ruling out all others, in stead, the challenge is to define the interactions between the various components and understand how modification of one factor will influence the other(s).

The complexity of mother-infant interactions has long been recognized by human developmental researchers [55] and goes beyond (just) the quantity of maternal care. Whereas in a large body of rodent literature, the physiological and behavioral outcomes of ES are often still attributed to alterations in maternal care [13,56-59], more and more preclinical researchers argue against the maternal mediation hypothesis [60] by acknowledging that variation in maternal care alone cannot explain all the effects of ES on HPA-activity and brain function [9,61]. Accordingly, as we proposed in chapter 1, early-
life experiences are modulated by multiple interacting variables that largely influence each other, including also (maternal) stress hormones and nutrient availability.

4.2 Essential Micronutrients in Early-Life

Despite the intense cross talk between the stress and metabolic pathways [62], the role of early nutrition has so far been largely ignored in the context of ES programming. In this thesis the role of (micro-) nutrient availability in the programming effects of ES was one of our main interests, for the following reasons. First of all, nutrition (in the form of maternal milk) provides the building blocks necessary for growth and development of the pup and also delivers the enzymes and cofactors needed for many biochemical processes. Notably, this includes the epigenetic machinery, which has been implicated in the lasting effects of ES (see section 5). Secondly, nutrition also conveys environmental cues about the pup’s (future) habitat. Thirdly, and most importantly, understanding how nutritional elements contribute to early programming can result in the development of novel nutritional intervention strategies, which are, in comparison with e.g. pharmacological treatments, relatively cheap, easily applicable and non-invasive.

Once we had developed a novel method to measure the micronutrient content of milk, plasma and hippocampal tissue of mouse pups (see chapter 3), we could test if ES alters micronutrient availability in the periphery and brain of the offspring. Moreover, we could study if nutritional intervention can exert beneficial effects on brain structure and function. In chapter 4 we showed that the limited nesting/bedding material paradigm not only elicited increased HPA-axis activity in pups, but also altered their nutritional status; specifically, it reduced methionine concentrations in plasma and brain. It remains to be fully elucidated how ES exerts this effect on the micronutrient status of the pups, but possibly ES: \( i \) diminishes the net intake of maternal milk; \( ii \) impairs the uptake of these nutrients; \( iii \) reduces bioavailability of these nutrients; or a combination of these factors. Interestingly, clinical studies suggest a positive association between high cortisol levels and high homocysteine levels (a hallmark of low vitamin B status) in healthy adults [63] and in patients with Cushing’s disease, which have very high cortisol levels [64,65].

Our findings that ES alters central and peripheral methionine availability and that restoration of methionine levels via dietary supplementation prevents some of the ES-induced cognitive impairments, support our hypothesis that nutritional elements are important for programming brain structure and function. Indeed, vitamin B₆ and B₁₂, folic acid, choline, betaine and methionine deficiencies are associated with altered brain development and function. Gestational folic acid deficiencies are further associated with reduced
proliferation and increased apoptosis in the fetal mouse hippocampus [66,67]. Similarly, insufficient maternal choline levels during gestation alter angiogenesis and DNA methylation patterns in the fetal mouse hippocampus [68,69]. Vitamin B deficiency during gestation and lactation has further been associated with increased apoptosis, disturbed neurobehavioral development [70] and impaired olfactory performance [71]. So far, most studies focused on the effects of methyldonor deficiencies during gestation (and sometimes also lactation).

In chapter 4, we show for the first time that a short period of ES-induced decreased methyldonor availability possibly contributes to the cognitive impairments in adulthood observed after ES-exposure, and that restoring these deficiencies via diet has beneficial effects. It is however remarkable that our diet does not seem to affect levels of neurogenesis neither postnatally nor in adulthood in male mice (chapter 4). However, we have a first indication that this might be different in female mice where MD-supplementation seems to boost proliferation in control as well as ES exposed adult mice (chapter 5).

4.3 ESSENTIAL MICRONUTRIENTS AND THE HPA-AXIS
Strikingly, restoration of the pup's nutritional status via maternal dietary supplementation prevented the increased HPA-axis activity in the pups (i.e. the rise in corticosterone and the associated adrenal gland hypertrophy), but did not prevent the fragmentation of maternal care. Thus, maternal care depends on the amount of nesting/bedding material in the cage, but not on methyldonor content of available food/water. This might be very different in studies addressing the effect of malnutrition or dietary insufficiencies; for instance, it has been shown that protein malnourished dams exert less licking and grooming behavior and show more often cannibalistic behavior than control dams [72].

Thus, while fragmentation of maternal care normally elicits increased HPA-axis activity in mice [10,73] or rat [74] pups, this no longer occurred in methyldonor-supplemented mice. This implies that methyldonor status can directly influence the HPA-axis. In chapter 4, we also showed that MD supplementation increased hippocampal GR expression in control pups. Increased GR expression in the control MD pups, which enhances glucocorticoid feedback sensitivity, could be a result of indirect regulation of adrenal function, or of the direct action of the diet in the brain. The fact that MD diet affected ES-induced circulating CORT without affecting GR expression would support the indirect trajectory. We could however not detect a significant reduction in CORT levels in control pups that were fed a supplemented diet when compared to those fed a standard diet. This could be due to a floor effect, as CORT levels in control pups are already very low and approach the lowest detection limit. Further research is
needed to unravel the mechanisms via which methyl donor supplementation modulates the HPA-axis.

Although quite some work has been done on effects of glucose and energy metabolism on stress hormones [75-77], much less is known on how methyl donors or related micronutrients and amino acids can affect HPA-axis functioning. For example, consumption of a protein rich meal in humans has been found to increase adrenal cortisol secretion [78,79], but since intravenous infusion of amino acids failed to elevate circulating glucocorticoids, this effect has been attributed to an indirect signal from the GI-tract [80] rather than a direct effect on adrenal function. In order to circumvent a possible effect of peripheral signals, future studies could employ central infusion of methyl donors in the hippocampus to address the direct effects of increased hippocampal methyl donor availability on HPA-axis activity [81]. How exactly micronutrient status affects HPA-axis activity remains unclear and await further investigations.

Another manner via which changes in methyl donor availability might affect circulating glucocorticoid levels is via epigenetic modification of genes regulating the HPA-axis. Because methyl donors are so important for the one-carbon metabolism and thus for DNA-methylation reactions, we tested if altered methyl donor availability results in altered DNA methylation status. Once we established that MD-diet did not drastically affect global DNA-methylation levels (see chapter 4), we specifically studied NR3C1 promoter methylation, as this GR-encoding gene can be epigenetically modified in the context of ES exposure and variations in maternal care, in rat hippocampus [82] and mouse hypothalamus [83], as well as in humans that were exposed to childhood stress [84,85] or to prenatal maternal depression/stress [86-88].

Importantly, several studies report that alterations in peripheral NR3C1 methylation status are induced by dietary conditions in perinatal life. For instance, increased choline intake in pregnant women is found to reduce fetal and neonatal circulating cortisol levels, by altering methylation status of CRH and NR3C1 [89]. In addition, protein restriction alters NR3C1 methylation in rat liver [90,91]. However, in line with our current findings on postnatal methyl donor supplementation (chapter 4), prenatal methyl donor supplementation was not found to alter the methylation status of the hippocampal NR3C1 in the offspring [92]. It remains to be investigated if NR3C1 methylation might be altered in other brain regions and/or in the periphery. Furthermore, it awaits further investigation whether other genes that (in-)directly regulate HPA-axis activity are epigenetically modified by methyl donor supplementation and if so, what determines the specific, and selective vulnerability of certain brain regions and genes to epigenetic programming by early nutritional components.
In conclusion, while there seems to be a bidirectional association between methyl donor availability and HPA-axis activity, the exact link between the two remains to be determined. Our findings do not allow us to conclude whether the beneficial effects of methyl donor supplementation on cognitive function are (exclusively) mediated via suppression of HPA-axis hyperactivity in early-life, or whether also other (direct) actions of methyl donors on neuronal plasticity (other than neurogenesis) and brain development are involved as well.

4.4 **Glucocorticoids, not the only mediators of ES effects**

Despite a suppressed HPA-axis activity, only some of the behavioral consequences of ES were prevented by methyl donor supplementation. This remains striking as most ES literature suggests that the programming effects of adverse early-life experiences are largely attributed to their influence on corticosterone levels. Glucocorticoids (GCs), are key in driving changes in gene regulation important for growth, development and adaptive programming in response to stress [93]. Thus, excess GC-exposure during sensitive developmental time-windows is likely to disrupt the normal developmental trajectories and may result in (an altered vulnerability to) pathologies later in life. However, we show that although the ES-induced rise in GCs was prevented, some ES-induced effects remained, including increased postnatal neurogenesis, reduced DG volume, reduced adult neurogenesis and hippocampus-dependent cognitive impairments in MWM probe and object location memory. This suggests that increased GCs levels are not implicated in all (lasting) ES effects and that some domains of cognitive functions might be more dependent on levels of circulating stress hormones than others.

Next to the role of environmental and nutritional elements, other important signaling molecules (not addressed in this thesis) could be implicated in early brain programming, for example: corticotrophin releasing hormone (CRH) and prolactin. In response to ES, expression of CRH increases in the hippocampus [94-96], and infusion of CRH into the brain of neonatal rats mimics some of the long-term ES effects, even when GCs levels are clamped at physiological levels [97]. In addition, antagonism of the CRHR1, but not GR, was shown to normalize hippocampal synaptic plasticity [98] and hippocampal function [94,95] after ES exposure. Furthermore, conditional CRF-R1 knockout mice subjected to chronic ES exhibited restored hippocampal plasticity and cognitive function [99]. Together, these findings strongly support the notion that CRH alterations are critically involved in early programming of hippocampal structure and function.

Another factor is prolactin (PRL), well known for its peripheral effects on the mammary glands to initiate and maintain milk production. It also exerts
central effects as it can reach the brain via receptor-mediated transport in the choroid plexus or local neuronal release in the hypothalamus [100]. PRL is implicated in the regulation of maternal behavior and its release and (hippocampal) receptor expression is particularly high during the postpartum period [101,102]. Interestingly, PRL is released in response to stress exposure, able to module HPA-axis reactivity and was shown to rescue hippocampal neurogenesis in chronically stressed adult mice [100,102].

Next to these examples, various other signaling molecules (e.g. cytokines, neurotropic factors, hormones, neuropeptides and nutrients) might play a role in early brain programming. Most of them are viewed as separate entities, whereas actually these factors are interrelated and their action might converge on the same cell and contribute in a synergistic manner to the consequences of ES. In neuroscience we often aim to model one single gene or molecule (in a specific cell type within a specific brain region) in order to study effects of a single factor on brain structure and function. While these reductionistic approaches help to understand the biological mechanisms contributing to brain function, an integrative approach might better represent the reality, where various biological processes in the organism are linked to critical components of its environment. Here, we made a first step by acknowledging that nutritional factors and their actions are embedded in a complex network of environmental and endogenous elements, which can synergistically affect the brain at multiple levels.

4.5 Maternal dietary intervention as a tool to prevent lasting ES-induced effects?
In chapter 4 we showed that nutritional intervention with a specific group of micronutrients during the ES exposure is able to prevent ES-induced cognitive impairments in adulthood. While, the lasting beneficial effects of early methyldonor supplementation on adult cognitive function have never been shown before, several studies using acute designs have shown restorative effects of methionine, methionine-choline or SAM supplementation on cognitive impairments [103-105]. For instance, lead (Pb⁺) exposure-induced learning and memory impairments in the MWM can be ameliorated by prolonged treatment with methionine choline (60 days) [104] or SAM (22 days) [105] in adult rats. In addition, cognitive deficits in contextual fear conditioning and object location, associated with epilepsy, could be reversed by a single I.P. methionine administration in adulthood [103], an effect associated with an increased methylation of the bdnf gene in the hippocampus, but without alterations in global DNA methylation levels. While the exact mechanisms via which methyldonor supplementation exerts its beneficial effect on cognitive functioning remain elusive, this confirms that methyldonors can restore cognitive abilities. As it was our goal to prevent ES-induced effects, by helping...
the organism to cope with the stress exposure, we restored methyl donor levels in the pups during the ES-period by increasing MD-content of the maternal milk. Below we will discuss the potential of this approach for clinical implications.

So far, studies testing the effects of maternal dietary intervention, have often focused on the role of a single nutrient. Classic examples are studies showing the importance of folic acid fortification during gestation to prevent neural tube defects. These studies have resulted in global recommendations for pregnant women to increase their folic acid intake [106]. Instead of fortification with one single nutrient, our postnatal nutritional intervention in mice enriched the maternal diet with several micronutrients, including folic acid, choline, L-methionine, zinc and B vitamins). While this approach does not allow to disentangle the separate contribution of each nutrient to the beneficial effects it is effective, possibly because all components of the 1-C metabolism interact and largely depend on each others availability as cofactors or substrates [107].

In the human population, nutritional deficiencies are often not restricted to a single micronutrient. In fact multiple micronutrient deficiencies are quite common, not only in developing countries (where they often result from inadequate dietary intake), but also in industrialized countries (e.g. as the result of a vegan life style) [108]. Therefore, improvement of maternal nutritional status during gestation and lactation (by improving the quality of maternal food or providing micronutrient supplements) is increasingly being recognized as a feasible evidence-based intervention to improve child health around the world [108]. As breastfeeding gives the most optimal health outcomes for both infant and mother [109] by facilitating the transmission of nutrients, hormones and cytokines from mother to child as well as through skin-to-skin contact, dietary supplementation of lactating mothers might also have more advantages than supplementation of the child alone. In case of maternal supplementation, both mother and infant will benefit, it is safer than supplementation directly to the child and it can supply the infant with the most bioavailable form of the nutrient [110]. Next to this, modified nutrition is non-invasive, home-based and relatively cheap and therefore also a very practical intervention.

So far it is unknown if stress exposure affects micronutrient status in humans. Recently, we started to study the relation between stress and maternal milk composition in humans. This might raise more awareness of the importance of key micronutrients in early life, especially in the stressful setting of the neonatal intensive care. Hopefully this knowledge will further improve clinical settings with the aim to reduce stress and optimize nutrition of both mother and child.
General discussion

Our current findings suggest that nutritional intervention is able to ameliorate ES-induced cognitive impairments in adult mice, even when it occurs only during a short period in early-life. If the postnatal period from P2-9 forms a critical time-window in which treatment has maximal efficacy, or whether an extended nutritional intervention would even have more drastic effects remains to be established. In the next section we will address the direct and lasting effects of ES on the brain and how this knowledge can help us determine critical time-windows for (nutritional) intervention.

5. MANIFESTATION OF ES EFFECTS: ACUTE AND LONG LASTING OR PROGRESSIVE?

We have shown that chronic ES impairs cognitive function in adulthood. We further questioned whether ES changes the brain early on, and acutely affects the developmental trajectory, or whether the changes develop more slowly, in a progressive manner. Are such ES effects then permanent or can they be prevented by treatment? If so, what would be the optimal time to intervene; should treatment occur in early-life, or can later-life interventions be as effective? In order to answer these questions we studied both the direct and lasting effects of ES on hippocampal structure and HPA-axis activity and we used two different types of intervention: nutritional supplementation during early-life (chapter 4) and exercise in late adulthood (chapter 5).

5.1 DISTURBED DEVELOPMENT OR COMPENSATORY RESPONSE?

A critical developmental period represents a time-window in which important organizational processes occur in a strict, well-timed order that can mostly not be reversed or repeated at a later time point. Therefore, disturbances during these time-windows generally have drastic and lasting consequences [111]. Indeed, while the effects of chronic stress exposure in adulthood are mostly reversible, e.g. after appropriate recovery times [112], the effects of chronic ES are found to be long lasting even when early alterations in bodyweight gain and HPA-axis activity have long been normalized.

As devised originally in the Baram lab, the limited nesting mouse model runs from P2-9 (actually, initiation of the model before P2 typically results in cannibalism by the dam and is therefore not feasible (Baram, personal communication). Modified versions of the model that run from P3-8 [113] or P8-12 [114] have been developed (in rats) and it appears that the timing and duration of the ES period critically determine which lasting effects are observed [11], most likely because of the different developmental processes that are at play at different postnatal time points. The period from P2-9, i.e. the ES period used in our studies, coincides largely with the critical developmental period during which the mouse hippocampal DG is formed. In the mouse, the
very first granule cells of the mouse DG are generated before birth (E17-22), but most of the DG is actually formed postnatally; at P5-7, proliferation in the DG peaks to generate the majority of the granule cells as well as the future neurogenic niche [115]. Its maintenance appears essential for life-long adult neurogenesis.

It is however important to realize that brain developmental trajectories not only differ between brain regions, but also across species. Mice are born with a relative underdeveloped hippocampus compared to humans; hippocampal development-wise, the first postnatal week in mice corresponds with the last trimester of gestation in humans [116]. Hence, the consequences of chronic postnatal stress exposure on hippocampal development in mice might be most comparable to consequences of prenatal stress exposure in humans. Admittedly, careful consideration of species differences is required in order to extrapolate our findings from mice studies to a human context.

In chapter 2 and 4, we showed that ES had both acute and lasting effects on the hippocampal DG. These included: a lasting volume reduction; an acute increase in developmental neurogenesis and a lasting reduction in long-term survival of developmentally-born neurons, as well as a (male-specific) reduction in the survival of newborn cells in adulthood associated with impaired cognitive function. While others have reported direct reductions in postnatal neurogenesis as a consequence of other forms of ES [117-119], several studies in rats have established a similar age-dependent biphasic effect of ES on neurogenesis with a transient increase in proliferation [120] and differentiation [121] in young animals and a lasting decrease in levels of proliferation and differentiation in adulthood [121-123]. Possibly, the biphasic nature of these effects can be explained by a depletion of the neurogenic stem cell pool due to the increased neurogenesis at younger ages (possibly as a compensatory mechanism to protect against the effects of ES).

Although we have not found reductions in proliferation at P150 (chapter 2) and P265 (chapter 4) in ES-exposed mice, it is possible that such effects are only detectable much later in life, and/or under specific conditions, such as exercise or specific challenges. We have addressed this to some extent in chapter 5, where we exposed the seemingly resilient ES-exposed female mice to running, a condition known to increase levels of neurogenesis. In fact, our preliminary indications suggest that ES reduces the proliferative response to running.

It is also possible that the difference in postnatal neurogenesis at P9 is not explained by an increase in the net number of proliferating cells in early-life, but rather by a deviation from the normal sequence of DG development.
Assessment of postnatal neurogenesis at multiple other time points within the first two postnatal weeks would be necessary to reveal how ES affects DG development and could demonstrate whether or not the increase in neurogenesis at P9 is the result of a delayed, mis-timed peak in developmental neurogenesis (which occurs around P5-7 under normal conditions).

The lasting effects of ES on behavior were profound at 5 months of age, a time point during which lasting DG volume reductions and a reduced survival of newborn neurons occur as well (see chapter 2 and 4). We did not address acute effects of ES on cognitive function in pups as this is practically challenging in animals this young, and rodents are not able to perform most tasks until they are weaned [124]. Although we did not study at what age the cognitive impairments appear for the first time, studies by others have indicated that cognitive impairments in ES-exposed mice are not present before 4 months of age (Baram et al. and L. Hoeijmakers, personal communication). In rats, the cognitive deficits were found to be prominent at 12, but not yet at 4 months of age [125]. Also from clinical studies it is known that a developmental insult may not manifest as impaired cognitive function until much later in life [124]. This is probably because the compensatory abilities of the brain diminish with age due to age-related reductions in various measures of neuronal plasticity. Indeed synaptic plasticity measures, such as LTP in CA3 and CA1 [125], dendritic complexity of CA1 pyramidal cells [125] and neurogenesis in the DG are reduced with age parallel to the ES-induced behavioral deficits. It would however be very interesting to determine at what age the ‘tipping point’ occurs, and what factors determine this. As we have shown, ES also affects DG development, prevention of the direct effects on DG structure might diminish the lasting effects on hippocampal structure and/or prevent the acceleration of age-related cognitive decline. In the next section we discuss the efficacy of such early interventions.

5.2 Timing of intervention; the sooner the better?

First of all, we questioned if nutritional intervention could support optimal brain development despite ES-exposure. We considered the possibility that the ES-induced reduction in methionine availability in the pups had induced a ‘delay’ in brain development. Omission of the essential amino acid methionine can limit protein synthesis, which is required for growth and formation of new cells, and which might hamper the formation of enzymes, neuropeptides and neurotransmitters, which convey important developmental signals. In addition, (protein) malnutrition in humans has been associated with reduced growth and delayed development of motor and learning abilities [126]. However, our 7-day postnatal dietary supplementation restored methionine levels at P9, but was not able to prevent the reduction in DG volume, nor the increase in postnatal neurogenesis at P9, indicating that either reduced
methionine availability was not (exclusively) responsible for these specific effects, or that our dietary intervention was not able to restore nutrient levels from the start of the ES period (as we only measured nutrient status at P9). Importantly however, our short and early nutritional intervention was already able to prevent some ES-induced cognitive impairments, suggesting that nutritional elements during the ES period can modulate the structural and functional consequences of ES. Whether nutritional intervention can also prevent ES-induced alteration in plasticity (other than neurogenesis) remains to be determined. Since interventions during the critical developmental time windows might not always be feasible, it would also be important to investigate if interventions later in life could be used to restore hippocampal structure and function.

Interestingly, opportunities for later-life intervention seem to exits, for instance during the (rat) pubertal period when also the response to stress later in life can still be (re-)programmed [127]. As another example, brief treatment with the GR antagonist mifepristone (during P26-28), which had previously been shown to normalize GCs-induced suppression of neurogenesis [128,129], was able to prevent the effects of maternal deprivation on neurogenesis [130]. Thus at least P26-28 likely represents a critical period during which several developmental processes are ongoing. As discussed in chapter 6, the adolescent period seems to be a sensitive period in programming later mental health [131,132].

As some of the ES effects do not occur until late adulthood, it would be interesting from a preventive point of view to address if the ES-exposed brain is still responsive to behavioral interventions during this phase of life. In a first attempt to answer this question, we studied in chapter 5 how ES affects neurogenic capacity in response to voluntary wheel running in late adulthood. The stimulatory effects of running on neurogenesis in rodents have been well-established [133,134]. They are present in males and females, usually maintained throughout the full life span [135,136] and are also effective in animal models of neurodegenerative disorders [137] or Down syndrome [138]. Interestingly, our data suggest that the (beneficial) effects of running are however absent in 8-month old ES-exposed female mice, which points towards a lasting effect of ES on adult neurogenic capacity in (late) adulthood. When considering the translational value of these findings one should keep in mind that the experience of running will be different between humans and rodents (especially those that are kept in captivity). However, this study was not meant to determine if intervention via physical activity per se could be beneficial but rather aimed to determine if behavioral interventions in late adulthood can still affect neurobiological processes in a brain that has been programmed in early-life.
In chapter 5, the early nutritional intervention seems more beneficial than adult exercise (as it increases neurogenesis in both ES and Ctl animals), but if this is due to i) the type of intervention, or ii) the timing of the intervention, remains to be established and it would therefore be interesting to also investigate the effectiveness of nutritional intervention during late adulthood. Together, our findings indicate that while the right interventions at the right time can ameliorate some effects of ES on the brain, ES exposure remains a powerful life event that programs some aspects of brain structure and function for life.

### 5.3 Epigenetics: Can Environmental Factors Lastingly Affect Gene Expression?

It remains intriguing how a relatively short period of stress can exert such long-lasting effects on later brain structure and function. In the last decade many studies have suggested that the early environment leaves a permanent epigenetic mark on certain genes, lastingy altering their expression which would result in profound changes in brain structure and function [82,139-143]. Initially, the epigenetic marks made in early-life (especially DNA-methylation) were considered to be very stable and therefore a likely candidate to lastingly affect gene expression and only certain interventions appeared to be able to reverse them [144] (e.g. pharmacological treatment with HDAC inhibitors [82], but also dietary folic acid supplementation [91] and central infusion with L-methioine [81]).

Nowadays however, more and more reports show that epigenetic modifications are more dynamic and can even be short-lasting or transient [145] and an initial event might induce a cascade of epigenetic modifications that can subsequently change with time. As an example, prenatal exposure to glucocorticoids induces very different changes in DNA methylation and H3K9 acetylation in guinea pig hippocampus when studied 24 hours or 14 days after treatment [146]. The mechanisms by which potentially dynamic epigenetic modifications in DNA and histone methylation states become stable remain largely elusive. In addition, we do not understand yet what is it that actually triggers these epigenetic mechanisms. And in addition to a full appreciation of their various consequences, what makes these modifications specific for certain brain structures? More so, what makes only certain genes susceptible for epigenetic modification, next to its nucleotide sequence (e.g. CpG density) that determines the capacity for epigenetic regulation [145]?

Whether epigenetic mechanisms are involved in the persistent cognitive impairments in mice exposed to the limited nesting/bedding paradigm was not elucidated. In chapter 4 we made a first step in this direction and found that ES and methylodonor supplementation did not lastingly affected global, or
NR3C1 specific DNA methylation patterns in the hippocampus suggesting that epigenetic changes are not strongly altered in this model. Further research is needed to reveal if epigenetics indeed play an essential role in mediating the lasting effects after ES in general and to understand what makes these ES changes model-specific.

6 SEX –SPECIFIC VULNERABILITY

The next issue we want to address is whether a sex-specific vulnerability to ES exists. Epidemiological studies have shown that sex is a crucial determinant for the effects of early-life experiences on the susceptibility to psychopathology. For instance, only male (and not female) offspring of mothers exposed to stress of the 1940 invasion in the Netherlands expressed increased risk of schizophrenia in adulthood [147]. In addition, effects of maternal depression during pregnancy on anxiety measures were more pronounced in 1-year old male compared to female infants [148], similar to adverse effects of parental separation during childhood on physical and psychosocial functioning [149]. As this could help us to understand how sex differences in the vulnerability to psychopathologies emerge, we were interested in finding out if sexual differences in ES-induced alterations exist at basal levels and/or after environmental challenges.

Although some preclinical studies had reported sex-dependent effects of ES [119,130,150-152], so far most animal studies did not investigate ES-effects in both sexes, as it requires large sample sizes to obtain enough power for statistical analyses. Most often it is considered more convenient to exclude females than to include synchronized females, or control for estrous cycle stage, and to adjust behavioral testing protocols for the assessment of both males and females in the same behavioral task. In chapter 2 we compared effects of ES in male and female mice and found that -while the direct effects of ES and the lasting volumetric changes were present in both sexes-, levels of adult neurogenesis and cognitive functions were differentially affected in males vs. females. It appears that females are more resilient to the effects of ES, possibly because they are i) less affected by fragmentation of maternal care and/or ii) protected by biological factors (see below). However, the preliminary findings described in chapter 5 indicate that ES-effects on levels of adult neurogenesis might just not be apparent in females under basal condition, but might become only visible at a later age (as for instance shown by [153]) and/or under conditions known to stimulate such plasticity. Moreover, in chapter 5, we provide indications that early MD-supplementation increases proliferation in females, hence it would be important to elucidate whether females are even more receptive to early nutritional intervention than males and which processes are responsible for these differences.
So far, it remains a question which mechanisms are involved in establishing sex-specific sensitivity to early-life experiences. Overall, levels of circulating sex steroids are relatively low in the offspring during gestation and in pre-pubertal life (see chapter 6). However, sex steroid exposure during critical time windows in perinatal development is crucial for the sexual differentiation of neuronal circuits (organization), that become activated in adulthood by sex steroids, resulting in sex-typical behaviours [154]. It is proposed that already subtle disturbances in sex steroid signaling during early development can disrupt the sexual organization of the brain and thereby induces sex-specific deficits in brain development and function [155] and differences in stress-sensitivity. For example, female rats that were masculinized within 24 hours after birth by injection of testosterone, exhibit a male-specific response to stress adulthood, in terms of spine density and learning [156]. This indicates that sex-specific programming of the stress response occurs already very early in development.

Thus early-life experiences likely have different effects in males and females because sexual organization of the brain has already taken place during prenatal and early neonatal life (by sex steroids and sex-determining region Y (SRY) gene expression during development, which is notably tightly controlled by epigenetic mechanisms [157]). Therefore the brain is already sexually dimorphic and can respond in a sex-dependent manner to early-life experiences, already before the levels of circulating sex steroids increase during puberty (see chapter 6). In addition, sex differences in the expression patterns of molecules important for epigenetic regulation might also be involved, e.g. females typically have higher levels of DNA methyltransferase enzymes and methylbinding proteins in the brain than males [158].

Another (obvious) possibility is that sex-differences in adult behaviour and neurogenesis are attributed to differential actions of circulating gonadal steroids in adulthood. As reviewed in chapter 6, anatomy of the hippocampus is sexually dimorphic and with its high abundance of estrogen and other sex steroid receptors, it represents a sensitive target for sex steroids. Interestingly, newly generated neurons also express estrogen receptors [159]. However, we do not completely understand yet how circulating estrogens modulate neurogenesis, as both estrogen-induced increases as well as decreases in this process have been reported [160].

We disprove the assumption that results from males also apply to females. Although a male bias is no exception in non-human biomedical research, especially in neuroscience [161], the relative neglect of females in (neuro-) biological research might have negative implications for women health, considering the prevalence of sex differences in disease susceptibility [162].
Therefore we underscore the major importance of investigating the biological basis of sex differences and inclusion of both male and female animals in neurobiological experiments.

7. ADAPTIVE SIGNIFICANCE, ANOTHER PERSPECTIVE?

After reading this thesis one is most likely left with the impression that the long-term consequences of ES exposure are always bad. Indeed from a neurobiological standpoint, dysregulation of normal ‘optimal’ developmental trajectories seems harmful. However, from an evolutionary perspective, this view is challenged: because ‘why would natural selection draft an organism to respond to adversity by becoming dysregulated’[163]? Throughout evolution, both supportive as well as stressful conditions have been encountered, thus one could argue that natural selection has crafted the organism to respond adaptively to both kind of contexts [163]. What we usually consider as a maladaptive deviation from the ideal developmental trajectory might actually be a very functional (adaptive) response given the later circumstances or context in which the organism finds him/herself. Evidence for this theory comes e.g. from studies showing that rats that received low levels of maternal care in early-life exhibit enhanced learning and memory abilities under stressful situations [59], despite alterations in various neuroendocrine and neuronal plasticity measures. Similarly, although ES induced by maternal deprivation affects dendritic morphology, neurogenesis and spatial learning abilities, ES-exposed rats showed improved emotional learning and hippocampal plasticity and function under high-stress conditions in adulthood, [122].

Interestingly, in an elegant study by Santarelli and colleagues, female mice exposed to aversive or supportive environments in early-life (limited nesting vs. handling) and in adulthood (single-housing vs. group housing), do not show completely opposite phenotypes when continuously exposed to aversive or continuously exposed to supportive conditions. In fact, the behavioral phenotype of mice with a mismatch between the adult and the early-life condition differed most from those with matched conditions [164]. Thus, elements from the early life environment seem to provide cues to the developing pups, preparing them for the environmental conditions that they are likely to encounter in adult life. This concept is known as the so-called match/mismatch hypothesis, which states that the better the anticipated environment matches the later encountered environment, the more optimal this preparation is and the better the individual pup will thrive.

Interestingly, this concept seems also applicable to the nutritional environment. For example, choline availability in utero (e.g. deficiency, sufficiency or supplementation) was found to determine spatial memory and hippocampal
plasticity dependent on choline availability in adult rats [165]. Exposure to a choline-deficient diet in adulthood impaired spatial memory in rats that were exposed to prenatal choline supplementation. While rats that received deficient choline in utero were unaffected by adult deficiency, adult supplementation in these animals actually impaired spatial memory and hippocampal plasticity [165]. In this perspective it would be interesting to test if mice that received MD-supplementation during the ES-period could further benefit from methyl donors supplementation in adulthood.

That mismatches between the developmental and the adult nutritional environment actually form a risk factor for poor health outcomes is evident from a large body of human epidemiological research. Eminent evidence for this comes from the ‘Dutch Famine studies’, that demonstrate the long-term health consequences in individuals that were prenatally exposed to the Dutch hunger winter, a period of severe famine in the Netherlands at the end of World War II [166]. These studies convincingly show that individuals who were initially exposed to under-nutrition in early-life, but faced with sufficient nutrition, or even over-nutrition later in life, have a higher risk to develop chronic diseases as an adult, including e.g. obesity [167], type 2 diabetes [168] and cardiovascular disease [169,170].

Not every individual suffers to the same degree from ‘mismatched environments’. It appears that two different traits exist: those who exhibit a highly adaptive capacity thrive in matched environments and those with a low adaptive capacity suffer from (accumulating) adversity (as proposed by the multiple-hit model [171,172]). It has been argued that this ‘differential plasticity’ evolves when the costs of a later mismatch exceed the benefits of being well matched [173]. Interestingly, highly reactive individuals (e.g. those carrying assumed plasticity alleles [174,175]) are likely to suffer more from adversity and to benefit more from supportive environments (in terms of mental health). It is tempting to speculate that reduced adaptive plasticity is what we observed in chapter 5, where we show that female neurogenic capacity is unaffected by ES under standard conditions, but can not be stimulated by exercise at an adult age.

In conclusion, accumulating evidence, from (pre-)clinical research and from the findings described in this thesis, support Barker’s hypothesis for a ‘developmental origin of health and disease’[176]. Thus if we want to improve the health of future generations, the prevention of adverse childhood experiences should start today. Based on our studies, a novel tool could be to reduce the impact of early-life stress via early nutritional interventions. This could possibly spare individuals, and thereby society, from an enhanced risk to develop psychopathologies and cognitive impairments in later life.
Chapter 7

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