Shaping the brain through experience: effects of stressful life events on hippocampal neurogenesis, morphology and function

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1. Summary
Numerous studies have shown that the hippocampus, both at a functional and structural level, is highly sensitive to stress and to the stress hormone corticosterone (McEwen and Magarinos, 1997, 2001; Joels et al., 2004). Stress is one of the major risk factors for stress-related pathologies like depression (Holsboer, 2000; Heim and Nemeroff, 2001; McEwen, 2003) and although depression is also characterized by a chemical imbalance of a.o. monoamine systems, more and more evidence points to additional involvement of changes in brain structure. Indeed, patients suffering from depression show a reduction in hippocampus volume, although it is not clear whether a smaller hippocampus is a cause or consequence of the disorder (Axelson et al., 1993; Rao et al., 2010). It is therefore an interesting question how stressful life events affect structural components of the hippocampus such as adult neurogenesis, and how this can further affect function in the context of vulnerability. For that reason, the overall aim of this thesis was to investigate effects of high-impact stressors (early life and chronic stress) on different phases of developmental and adult neurogenesis, the long-term functional consequences of these events, and the possibility to pharmacologically intervene.

Main Findings of this thesis:
- Stress early in life selectively affects neuronal maturation during development and has opposite effects in males and females (Chapter 2).
- The long-term consequences of early life stress exposure on structure, point to irreversible changes in the female offspring, where reduced numbers of granule neurons in the dentate gyrus were observed (Chapter 3).
- Long term effects of early life stress exposure on structural plasticity, i.e. a reduction in adult hippocampal neurogenesis, were found in males only (Chapters 3 and 4).
- At the functional level, effects of early life stress are most pronounced in male offspring; males show an increased long-term potentiation in the presence of the stress hormone corticosterone, and an enhanced emotional memory. In contrast, spatial learning ability was impaired (Chapters 3 and 4).
- Exposure to chronic unpredictable stress in adulthood reduces the level of adult neurogenesis and newborn cell survival, which is reversible by short-term treatment with the GR antagonist mifepristone (Chapter 5).

In chapter 2, it was shown that early life stress by 24h maternal deprivation (MD) at PND 3 alters hippocampal structural plasticity in a sex-dependent manner in 3-week old rats. Maternal deprivation increased corticosterone levels at PND4, but did not have immediate effects on cell proliferation in the dentate
gyrus. At three weeks of age, newborn cell survival and proliferation rate were not changed by MD at PND 3. However, neurogenesis as measured by the number of doublecortin-positive, immature neurons, was increased in male but decreased in female offspring. No such differential changes were found in granular cell layer volume, total astrocyte number or gliogenesis, indicating that early life stress elicits sex specific changes in a subset of the differentiating cell population in the developing animal. Careful observations of the amount of maternal care indicated that overall, male pups received significantly more care than female pups. In response to maternal deprivation, the dam increases licking and grooming behaviour, in a comparable manner towards both sexes, so that the differential effects on neurogenesis are unlikely to be caused by changes in maternal behaviour per se.

To investigate the consequences of the decreased population of immature neurons during development in females, we studied in chapter 3 the long term effects of 24h MD at PND 3 at a structural, functional and behavioural level in adulthood. MD-exposed females had a lower total neuron number and density in the dentate granule cell layer and displayed significant differences in the dendritic arrangement of granular cells, indicating that early life stress in females has lasting and irreversible structural consequences. No effects of MD were found on hippocampal neurogenesis in adult female rats, as was studied at the level of cell proliferation, survival and immature neurons. As the decrease in neurogenesis at PND 21 does not last into adulthood, it may represent a transient developmental phenomenon possibly resulting in the observed reduction in total granule cell numbers at a later age. At a functional level, this structural change after MD had (surprisingly) little effect on synaptic plasticity in the dentate gyrus and on hippocampus-dependent learning tasks in young adult females. There was no indication that MD alters general anxiety in adult female rats, as measured by an elevated plus maze, however, we did observe an improved amygdala-dependent fear memory after MD. In summary, we conclude that early maternal deprivation in females has lasting effects on dentate gyrus structure, with little consequences for hippocampal function.

Similarly, to follow up on the increased number of immature neurons in male offspring (chapter 2), we also studied long-term consequences of MD in adult males, as reported in chapter 4. Severe early life stress resulted in a lower level of cell proliferation throughout the dentate gyrus and lower numbers of adult generated, 3-week old cells and of young neurons in the caudal part of the hippocampus. In addition, we found changes in the dendritic arrangement of individual granule cells, neurons showed a reduced dendritic complexity in the proximal part of their dendritic trees, but total granule cell number was not affected. At a functional level, animals showed impaired spatial acquisition, whereas contextual and cued fear memory formation was improved. This was paralleled by an enhanced hippocampal synaptic plasticity in vitro in the presence of corticosterone. Thus, extensive structural and functional adaptation occurs in the hippocampus following MD at PND 3, which leads to improved functioning in later life, but only in a stressful context.
Chapter 6. General Discussion

In chapter 5, we studied the effects of exposure to chronic, unpredictable stress in adulthood, representing another form of a high-impact stressor, on different phases of hippocampal neurogenesis. We found that neurogenesis was reduced after 21 days of unpredictable stress at the level of newborn cell survival and immature neurons. The cell population labelled by BrdU at the first day of the stress regimen, as well as doublecortin positive neurons, were reduced in number. For cell proliferation, no differences between the groups were found. Interestingly, short term (4 day) treatment with the GR-antagonist mifepristone at the end of the stress regimen, was found to normalize the stress-induced reduction in BrdU and doublecortin positive cell numbers. The drug alone, i.e. in the absence of stress, did not affect neurogenesis.

2. Methodological considerations

The exact experimental design, choice of model and techniques used can influence the results of a study and thus explain differences between studies. I will therefore here discuss the designs we have chosen for our studies as well as some of the possible draw-backs and limitations of our experiments and the factors that should be considered when interpreting the results.

2.1. Stress pathology: choice of models

When studying stress in animal models in the context of stress pathology, some important (although at first glance trivial) questions should be asked. First of all, does the model induce (sufficient amounts of) stress? Second, is the control group not stressed? Third, are there any (external or internal) compensatory or counteracting mechanisms initiated or does the stress response adapt to this type of stress? In the next section, I will address some of these questions and explain the rationale behind the models used in this thesis: early maternal deprivation and chronic unpredictable stress.

Early life stress models: components of the early life environment

As mentioned in the introduction, maternal separation protocols differ considerably between different labs and studies, regarding the level of stress severity, the timing of stress exposure and the control group(s) against which the results are compared (Pyce and Feldon, 2003). This is probably one of the major contributors to the variation in the current literature. An important consideration in early life stress models is the relative contribution of, on the one hand, postnatal stress levels in the pups and on the other hand stress levels in the dam that can induce changes in maternal behaviour or result in increased corticosterone levels through lactation, which should be considered in both the experimental group and the control group (Macri and Wurbel, 2006).

Maternal absence is one of the early life stressors that is likely to occur in a naturalistic context, where the dam may be forced to leave the nest in order to find food or due to environmental threats. In principle, the most favourable environment would be a completely undisturbed environment, without threats
and with a minimum time of maternal absence. When the rate of disturbance between dam and pups increases, the early life environment becomes more and more adverse. However, the level of maternal care has actually been shown to increase in protocols like early handling and maternal separation, which is triggered by nest disturbance (chapter 2, Francis and Meaney, 1999; Pryce et al., 2001), since upon return to the nest, the dam provides a bout of maternal licking and grooming. Whereas minor disturbance induces higher levels of maternal care, severely stressful situations probably force the dam to provide less care, eventually. In conclusion, a completely undisturbed early environment is not necessarily the most favourable, and an increasing environmental threat is compensated for at the level of maternal behaviour. The ‘optimal circumstances’ for pups therefore seems to be an environment challenging enough for the mother to show increases in maternal care, without evoking high stress levels in the mother or in the offspring due to prolonged maternal absence (Pryce and Feldon, 2003), figure 1.

![Diagram showing maternal care and stress levels in response to environmental adversity](image)

**Figure 1.** With increasing environmental adversity, both maternal care (dashed-line) and stress levels in the mother (black line) as a result of environmental stress increase. As for the amount of adversity experienced by the offspring (grey line), which determines e.g. stress responsivity later in life, this is likely to follow an inverted U-shaped distribution. Adapted from Macri and Wurbel, 2006.

With this in mind, the choice for a suitable maternal deprivation model and proper control groups is critical for the outcome of the study. In this thesis, we have studied the model of 24h MD on postnatal day 3 using the whole-litter 146
method. The rationale was to maximally impact dentate gyrus development by a severe single-episode stressor at the beginning of the stress hyporesponsive period, as this period coincides with dentate gyrus development (see chapter 1, figure 2). In our first study (chapter 2) we also tried to counteract effects of food deprivation due to maternal absence. For this, maternally deprived animals received high dose glucose injections during deprivation, which has been shown to slow the onset of the HPA-axis response in maternal separation in mice (Schmidt et al., 2006). A second group of maternally deprived animals received sham injections (without injection volume) and two control groups that stayed with the dam were used: sham injected and undisturbed (the latter not being culled or cage-cleaned). At the end of the maternal deprivation period corticosterone levels in the two MD groups were increased compared to both controls, indicating that the SHRP was disrupted due to MD. Administration of glucose could not prevent this. Also, no differences were found in corticosterone levels between the undisturbed control group and the sham injected control group (arguing against a relative “stressful” environment in undisturbed litters), although it should be noted that the undisturbed group did receive a subcutaneous BrdU injection 22 hours before blood sampling. In conclusion, 24h of maternal deprivation at PND 3 induces higher corticosterone levels which is not prevented by glucose administration. The follow-up studies in chapters 3 and 4 were therefore performed without the inclusion of an undisturbed and glucose-injected group.

Regarding the levels of maternal care in response to our MD protocol (comparing control-sham with MD-sham), maternal deprivation on PND 3 increased licking and grooming (LG) of male and female pups on PND 4 (chapter 2). This increase is significant compared to the other postnatal days and compared to the control-sham group that received three 10-minute handling sessions on PND 3 (figure 6, chapter 2). Both in control and MD treated litters, females received less care than males: a difference that was more pronounced in the second half of the first postnatal week. The increase in maternal care due to MD however, was significant in both sexes. Also, we found that dams increase the level of active (arch-back) nursing in response to MD, during the remainder of the first postnatal week (figure 7, chapter 2). In conclusion, it appears that long-term separations like MD induce a short term increase in LG and a more persistent increase in ABN, but this affects male and female offspring to a similar extent.

Another putative effect of MS or MD, besides changes in maternal care, is a potential increase in stress hormone levels of the dam that may be transferred to the pups via the milk. Lactation is paralleled by higher then normal corticosterone levels (Zarrow et al., 1972) and lactating dams are relative unresponsive to normal stressors (Windle et al., 1997; Neumann, 2001), but do show a further rise in corticosterone after ‘pup-related’ stress (Smotherman et al., 1977). Although we did not measure maternal corticosterone levels in our study, repeated separation was found to increase corticosterone levels, to a
greater extent in short-term then in long-term separations (Eklund et al., 2009). These increased levels may reach the pups through the milk, possibly adding to the effects of increased pup corticosterone levels in response to repeated separation itself (Domenici et al., 1996; Catalani et al., 2000).

In conclusion, changes in maternal behaviour and maternal corticosterone levels that occur after handling and/or separation, may add to the effects of increased stress hormone levels in the pups, although in the current model these effects are confined to a single episode. Increased maternal after return of the pups to the nest care might partly compensate the effects of separation. However, considering the duration of the stress (24 h) and the subsequent lasting effects on a.o. hippocampus structure, it seems that increases in maternal care can not (fully) normalize the increased corticosterone levels due to maternal deprivation.

*Chronic stress models*

The chronic stress protocol used in this thesis was adapted from Herman et al, 1995 and consists of different types of both physical and psychosocial stressors, administered twice daily and alternated in a random fashion for a period of 21 days. This was done to avoid habituation to stressful events, as was reported before to occur in protocols of, for example, repeated restraint stress (Magarinos and McEwen, 1995; Garcia et al., 2000; Marin et al., 2007). The present chronic, unpredictable stress model has been shown to increase basal corticosterone levels (Herman et al., 1995) although not in all studies, which is probably due to a large individual variation (van Gemert and Joels, 2006). The latter study did however report a reduced body weight gain and an increased adrenal gland size, both hallmarks of a hyperactive HPA axis (van Gemert and Joels, 2006). In addition, the same protocol induced stress, that altered both hippocampal structure and function (for a review see Joels et al., 2004; Joels et al., 2007). Regarding the interpretation of our current results, the GR antagonist mifepristone (or vehicle) was administered orally at the end of the stress period to both stressed and control animals. This may be stressful in itself and should thus be considered when comparing the results of chronically stressed animals with the control group (see section 3.2.).

### 2.2. Measuring the effects of stress: choice of techniques.

Regarding the analysis of neurogenesis, a couple of important issues have to be considered. When determining differences between numbers of newborn neurons in a control versus a stressed group, it is important to realize that there is no universal or unique marker for neurogenesis. In order to gain information about multiple phases of this process, we used three markers to measure neurogenesis in the experiments described in this thesis. The endogenous cell cycle marker Ki-67 was used to identify proliferating cells; the exogenous birth date marker BrdU was used to label cells born 3 weeks before the end of the
experiment and the microtubule associated protein doublecortin (DCX) was used to identify immature neurons (chapter 1, box 2).

The use of BrdU has accelerated the field of adult neurogenesis research as it not only allows non-radioactive identification of newborn neurons but can also be combined with other types of immunohistochemical labelling to reveal the phenotype of the newborn cells. However, quantification of BrdU-positive cells is influenced by several factors. First of all, when BrdU is used to measure (long term) cell survival it may dilute below detection level, especially in neural stem cells or progenitor cells that do not commit to lineage, but continue to divide (Prickaerts et al., 2004). Since neural stem cells are a relative small population and their progeny, the transiently amplifying neural progenitors commit to lineage on average within 2-3 cell divisions (when BrdU is still detectable), label dilution most likely does not have a great effect on the mere numbers. Hence, the majority of BrdU-positive cells is still visible after 3 weeks (Kempermann, 2006, chapter 6). Second, being a thymidine analogue, BrdU may also be incorporated in the DNA during DNA-repair mechanisms and possibly create false positives. This concept may e.g. also apply to cells undergoing apoptotic cell death. Radiation studies that induce DNA-damage have shown that BrdU involvement in DNA-repair processes is very limited and does not play a significant role in the normal adult brain (Cooper-Kuhn and Kuhn, 2002; Mizumatsu et al., 2003). Also, labelling with viral vectors identifies positive cells in the exact same locations and with similar frequencies as after the incorporation of BrdU (van Praag et al., 2002; Eisch and Mandyam, 2007; Kuhn and Cooper-Kuhn, 2007).

Besides these issues, the doses of BrdU applied deserves consideration. On the one hand, a low dosage may not be able to label all dividing cells and thus lead to an underestimation, whereas too much BrdU may be toxic, as supported by results from in vitro studies (Caldwell et al., 2005). In adult animals a dose up to 250 mg/kg is recommended (Kempermann, 2006), which was shown to label all cells in S-phase, without causing toxicity (Cameron and McKay, 2001). The problem of toxicity seems to be less of a concern in adult animals, as even higher doses in the rat did not interfere with hippocampal cell proliferation and neuronal maturation (Hancock et al., 2009). Toxic effects of BrdU are probably mostly restricted to BrdU-labelling during embryonic development (Kolb et al., 1999). When BrdU is injected in pregnant females, their offspring indeed shows a disrupted development, i.e. bodily defects (Biggers et al., 1987; Kolb et al., 1999) but this was virtually absent after postnatal injections (Kolb et al., 1999). Toxic effects of BrdU further depend on dosage, timing and specific brain region (Kolb et al., 1999; Kuwagata et al., 2007) and the effects of early BrdU exposure on the brain may be largely determined by the permeability of the blood brain barrier, which is higher earlier in development (Xu and Ling, 1994). These observations are relevant for Chapter 2 where BrdU was injected at PND 3. In order to avoid toxic effects we used a lower dose than the study of Kolb et al (1999), a dose that did not yield
any abnormalities in earlier studies (D. Inta, Institute of Mental Health, Mannheim, personal communication).

BrdU is incorporated by cells within approximately 2 hours after injection and then cleared by the liver, which greatly facilitates pulse-labelling. However, the number of cells found in BrdU labelled tissue depends on how many cells incorporate BrdU at the time of labelling, how many of these cells subsequently proliferate and/or how many of these cells eventually survive. Although in studies using BrdU-labelling the term “survival” is often used, the number of cells represents actually both proliferation and survival rate. How this may have influenced the results from this thesis, is in detail discussed in section 3 of this chapter.

The other markers used in this thesis, i.e. Ki-67 and doublecortin (DCX) have their own specific properties and with that, advantages and disadvantages. Ki-67 represents the number of cells in cell cycle, which lasts for 24 hours on average. Therefore Ki-67 analysis is very sensitive to manipulations that may have occurred during the last day before collection of the brain tissue. Also, similar to BrdU, Ki-67 alone does not provide any information about the fate or later phenotype of newborn cells, as this requires double labelling with other markers. Doublecortin is expressed in immature cells committed to neuronal lineage and therefore provides information about the rate of neurogenesis (Meyer et al., 2002). A subpopulation of these cells is able to re-enter a proliferative state (Brown et al., 2003; Kronenberg et al., 2003). Since doublecortin is expressed in cells up to 2-3 weeks after proliferation the number of DCX-positive cells represents neurogenesis during a rather large time-window. A recent study identified differential stages of maturation within the doublecortin population (Plump et al., 2006), see also chapter 1, box 3. This classification proved also useful for the studies described in this thesis, as stress affected different sub-populations of DCX cells to a different extent (chapter 4).

Regarding the issue of quantification, information about the number of newborn cells, glia cells or granule cells (and for example volume estimates) has to be inferred from two-dimensional sections, which is referred to as stereology. Estimating total cell populations should be performed by sampling in an unbiased yet systematic and random fashion, throughout the structure of interest. In our experiment, stereological quantification was carried out with the Stereoinvestigator system (SI, Microbrightfield, Germany). Provided that serial sectioning is performed systematically (cutting brains in a 1 in 10 series, for example), the SI-software ensures random sampling throughout the surface area of each section and in the z-direction i.e. focal plane of the section (for more information, see www.stereology.info/optical-fractionator/). Although this form of sampling provides unbiased cell numbers, other factors are worth considering such as total granule cell numbers, which allows us to express the rate of neurogenesis as a percentage of the total GCL population.

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3. How do stressful experiences shape the brain?
The experiments presented in this thesis show that stress changes hippocampal structure, but that effects are highly dependent on the type of stressor and the animal that is stressed. For example, maternal deprivation can induce structural changes, which can be very different even between littersmates; something that solely depends on the sex of the animal. Also, the consequences of a single stressor early in life reduces neurogenesis up to 10 weeks later, whereas neurogenesis in adulthood is reduced after 21 days of stress but can be reversed by application of a glucocorticoid receptor antagonist for just four days. Although our data confirm that stress can affect adult neurogenesis and change structural parameters, there are still inconsistencies among our and other studies, which are most likely due to variation in stress protocols, the timing of BrdU labelling or the specific phase of neurogenesis or structural parameter studied.

In chapters 2, 3 and 4, we studied effects of early maternal deprivation on hippocampal structure. During the early postnatal period, the brain undergoes rapid growth as characterized by high levels of cell proliferation and the formation of neuronal connections. This is especially true for the dentate gyrus of the hippocampus (see box 2, Introduction) that develops for a large part postnatally. Therefore, stress during development may exert long lasting effects on the brain, especially since the HPA axis is already responsive by day 15 of gestation (Fujioka et al., 2003) and the GR is present in hippocampal cells in the foetal brain from day 13 of gestation onward (Cintra et al., 1993).

One of the most striking results after early life stress is the dichotomy between males and females. This may be relevant in the context of the sex-dependent vulnerability to e.g. depression. Although we did not see any sex differences in stress-hormone levels or hippocampal cell proliferation immediately after maternal deprivation (chapter 2), MD induced an increase in the number of immature neurons in males versus a decrease in females at 3 weeks of age. In adulthood, females show a decrease in the number of granule cells of ~15%, which was absent in males. Starting with these seemingly lasting effects of MD on dentate gyrus structure in females, it is possible that the decrease in the number of immature neurons in 3-weeks old females and the reduced total granule cell population in adulthood are causally related. Apparently, although a rise in corticosterone at PND 3 does not result in immediate inhibition of proliferation, it does slow or inhibit subsequent neuronal maturation as it has resulted in a smaller dentate gyrus at a later age. This is consistent with an earlier finding reporting a sex-dependent effect on the number of granule cells as a result of subject to prenatal restraint stress (Schmitz et al., 2002; Zhu et al., 2004); granule cell numbers were reduced only
in females, and not in males. From this it can be concluded that early life stressors preceding or coinciding with dentate gyrus development can have lasting effects on granule cell numbers, but mainly in females.

In males, early life stress did not affect total granule cell numbers, but did alter structural plasticity (chapter 4). Hippocampal cell proliferation in 69-days old animals was reduced, as measured by the number of Ki-67-positive cells. This is probably not directly related to differences in corticosterone levels, since no changes in baseline corticosterone, nor stress response were found although we have not enough data points to exclude differences in circadian or ultradian rhythms. It is not unlikely that the reduced proliferation rate occurring at day 69 is already present in 31-days old animals, especially since 3-weeks old males subjected to MD (in combination with glucose injection) already show reduced proliferation levels (chapter 2). If so, this would imply that the number of cells incorporating BrdU on PND 31 was lower in MD treated animals. From this, it may be expected that MD animals have less BrdU-positive cells at PND 69. At first glance, we found no differences in the number of BrdU-positive cells, suggesting compensatory changes at the level of cell survival. After closer examination, an effect along the rostrocaudal axis was revealed. Males with a history of early life stress have less 3 week old cells in the caudal part of the dentate gyrus. This was confirmed by DCX cell counts; MD males also show a lower number of DCX-positive cells in the caudal but not in the rostral part of the dentate gyrus. The more immature DCX phenotype (types 2 and 3) were reduced in number, consistent with the effects of early life stress on proliferation.

Furthermore, reduced cell proliferation in males did not lead to a reduction in the total granule cell number, which might have been expected at least in the caudal part of the dentate gyrus. Of course, it is possible that males develop a smaller granule cell layer later in life. However, considering the possible compensatory effect on survival in the rostral dentate gyrus, an interesting alternative explanation may that the hippocampus of male offspring is endowed with a neuroprotective capacity. Other studies have suggested the occurrence of neuroprotective mechanisms after exposure to early life stress. For example, it was observed that immediately after maternal separation, only males show an upregulation of calcium-binding proteins in the hippocampus, providing a neuroprotective mechanism (Lephart and Watson, 1999). Also, an earlier study that did not find effects of maternal separation on cell survival, did report an increase in BDNF protein levels in the hippocampus in males, and proposed that this reflected a compensatory mechanism (Greisen et al., 2003).

In conclusion, the differential spatial distribution and possible neuroprotective effects in males may explain why others, despite an inhibitory effect of MD on proliferation, failed to find differences on subsequent survival (Mirescu et al., 2004).

*Explaining sex differences after early life stress*

The underlying cause of the dichotomy between male and female offspring is probably a complex interaction between differences in development due to sex
hormones with increased corticosterone levels due to MD. Sex hormones during development create life long differences in the organisation of the neural circuitries including those involved in stress sensitivity (Goy and McEwen, 1980; MacLusky and Naftolin, 1981; Patchev et al., 1999). Hence, differences due to our postnatal manipulation may be explained by (1) differential interactions between stress hormones and sex hormones in early life, or (2) through a difference in the speed of brain development between males and females (resulting in a different developmental phase at the time of stress exposure), for example. Which of these specific mechanisms or alternatively whether a combination of the two is responsible for the differences between male and female structural maturation is unknown, but the outcomes of studies using prenatal stress elucidate at least some of these aspects.

First, supporting a role for differences in speed of development between males and females, exposure of humans to stress during the second (but not first) term of pregnancy induces a higher incidence of schizophrenic symptoms and major depression in men only (van Os and Selten, 1998; Watson et al., 1999). It is possible that the increased vulnerability in men in this case, is related to the timing of stress exposure relative to the developmental phase, as the degree of vulnerability to the effects of prenatal stress depends on the time of exposure (Yehuda et al., 2005). Moreover, the speed of cerebral development is different in men compared to women (Taylor, 1969). In support of a role for a sex-dependent interaction between sex and stress hormones, prenatal stress research in animals demonstrated a distinct role for sex hormones in the effects of stress on brain and behaviour later in life. Prenatal stress induces demasculinization and feminization in males and females respectively (Bowman et al., 2004; Weinstock, 2007; Mandyam et al., 2008). At day 18 and 19 of gestation, testosterone levels and aromatase activity induce sexual differentiation in terms of brain development (McEwen et al., 1977). Prenatal stress is able to interfere with this, inducing a more feminine phenotype in the male offspring subjected to prenatal stress (Ward, 1972; Ward and Weisz, 1980; Jimbo et al., 1998). In rats, the actions of sex hormones can be detected during the last week of gestation and the first ten postnatal days (MacLusky and Naftolin, 1981) indicating that during MD on PND 3 sexual differentiation of the brain is in progress.

Sex hormones stimulate neurogenesis, also in the perinatal period. For example, estrogens have neuroprotective effects (Garcia-Segura et al., 2001) and e.g. administration of estrogens to female pups results in a larger dentate gyrus (Drekin et al., 2009). Also, testosterone was shown to increase cell proliferation in the brain of neonatal rats (Seress, 1978) and enhances cell survival in adult rats (Spritzer and Galea, 2007). Serum concentrations of testosterone from PND 1-10 are about 3-fold higher in male rats than in females, whereas the serum levels of estrogens do not differ (Pang et al., 1979). This may explain why males have larger hippocampi and more neurogenesis than females during development (chapter 2, Hilton et al., 2003; Zhang et al., 2008). In addition, the DCX cell population which was differentially affected on PND 21, could be
under the regulatory control of sex hormones as estrogen receptor β is present on DCX-positive cells during the neonatal period (Herrick et al., 2006). In songbirds, testosterone administration could indeed increase the number of DCX-positive cells (Balthazart et al., 2008). Interestingly, a recent study further showed that males compared to females have more neurogenesis in the dentate gyrus and CA1-region at PND 4, while administration of testosterone to female pups resulted in an increase in neurogenesis up to the level of the males (Zhang et al., 2008). Therefore, it is possible that in males, the effects of a rise in corticosterone on PND 3 is compensated for, or counteracted by high testosterone levels, explaining the increase in the DCX-positive cell population whereas in females, this protective potential is absent and a subsequent reduction in the number of cells is found.

The differential effects of early life stress on hippocampal structure persist into adulthood. In males a reduced cell proliferation was found, whereas in females there are no effects of MD on adult neurogenesis (chapter 3 and 4). The lack of effect in females may be due to differential programming effects of early stress exposure or may be secondary to lower levels of baseline corticosterone in adult females (chapter 3), since this was shown to increase newborn cell numbers (Cameron et al., 1993). In addition, differential regulation of neurogenesis in response to stress is known to occur in females; chronic stress was even shown to increase survival of newly generated neurons (Westenbroek et al., 2004). Finally, progenitor proliferation fluctuates across the oestrous cycle (Tanapat et al., 1999; Tanapat et al., 2005), which potentially could have induced subsequent variations in cell numbers, preventing us from detecting the reduction in Ki-67 positive cell numbers after MD. However, arguing against this, we found no differences in variation between males and females in this parameter.

In conclusion, the effects of early life stress on adult hippocampus structure differ between males and females. This is possibly mediated through the interaction between sex and stress hormones during the early postnatal period, or due to the neuroprotective effects of, for example, testosterone. Males show reduced levels of adult neurogenesis; a process still under the regulatory control of the environment, whereas females show more lasting changes in overall dentate gyrus structure, i.e. adult cell number, which could possibly render them more vulnerable later in life. How this may affect animals at the functional level will be discussed in section 4 of this chapter.

3.2. Effects of chronic stress in adulthood on neurogenesis

In chapter 5 we found that chronic stress in adult males reduces the number of BrdU- and DCX-positive cells without affecting cell proliferation. In the literature, reductions in proliferation or neurogenesis are commonly reported after chronic stress according to several (Czech et al., 2001; Pham et al., 2003; Heine et al., 2004b; Dagyte et al., 2009), but not all (Lee et al., 2006; Snyder et al., 2009) studies. The lack of effect in chapter 5 may be explained in several
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ways. First of all, when assuming that stress reduces cell proliferation (directly or indirectly) through increased corticosterone levels (Cameron and Gould, 1994), a prerequisite is that animals actually have increased hormone levels during the 24 hours before sacrifice (i.e. the expression window of Ki-67). In our case, the last stressor was applied 16 hours before perfusion, which (time-wise) allows interference with the proliferative population. However, since we did not measure stress-induced corticosterone levels in this specific cohort of animals, we cannot exclude the possibility that this last stressor may have failed to cause a significant rise in corticosterone. Neither do we know whether animals from this cohort suffered from elevated baseline corticosterone levels at some point during the last day. Despite this, the model used in our study induced a hyperactive HPA-axis and minimized habituation to stress (Herbert et al., 1995). It is therefore likely that either baseline or stress-induced levels are indeed elevated at some point during the last day. In that case, a second possibility is that increased corticosterone levels only affect proliferation rates for a short period of time. Indeed, 16 hours between stress and perfusion may be too long according to Dagyte et al. (2009), who reported that chronic footshock stress decreased proliferation 2 hours, but not 24 hours after the last stressor. A study by Heine et al. (2004b) however, applying the same chronic unpredictable stress protocol as used in our current study, did report a reduction in proliferation that only partially recovered even after 3 weeks without stress. Therefore, a third possibility is that in our study both control groups received either vehicle (milk) or mifepristone during the last 4 days of the stress regimen. This, in itself is likely to cause a rise in corticosterone and could cause a reduction in the number of Ki-67 positive cells to the level of the stress group.

Of course, it is also possible that a stress-induced increase in corticosterone simply does not, under all circumstances, inhibit cell proliferation as was reported by some studies using a single stressful episode (Pham et al., 2003; Thomas et al., 2006; Thomas et al., 2007; Dagyte et al., 2009) and even in some cases after repeated stressors (Lee et al., 2006; Snyder et al., 2009). This suggests that a rise in corticosterone level may be necessary but not sufficient for the effects of stress on cell proliferation. The effects may depend on the type of stressor, species, strain, age, sex or other factors. In addition, especially during the transition from ‘acute’ to ‘chronic’ stress exposure, changes in other factors related to HPA-hyperactivity like CRH levels, changes in MR or GR expression, may play an important role in the final outcome (de Kloet et al., 2005). Curiously enough, some stress protocols show a more pronounced effect after a single episode, but yield smaller effects when studied in a chronic setting (comparing Gould et al., 1997 and Czeh et al., 2001). Others however, find that the effect of a single stressor is less pronounced when comparing it to chronic stress (Pham et al, 2003; Dagyte et al, 2009, Heine et al, 2004b). The first phenomenon may also be due to habituation at the level of the HPA-axis, i.e. repeated stressors may fail to induce elevated stress hormone levels at a certain point, whereas the second observation may represent a progressive disturbance
of homeostasis at a neuronal level, through adaptation of receptor expression levels or chronic changes in growth factors.

The reductions in BrdU- and DCX-positive cell numbers after chronic stress are in agreement with each other, although these markers represent different, but partially overlapping cell populations. In our study, cells were labelled with BrdU at the first day of the 21-day stress regimen, 2 hours after the first stressor. Therefore, we are unable to isolate possible effects of stress on proliferation per se from the mere survival that occurs later. The first stressor may have reduced proliferation, thereby reducing the number of cells that incorporate BrdU. The timing of BrdU-labelling may thus explain the differences between chapter 5 and the study of Heine et al (2004b), where no effects of stress on cell survival were found, despite the use of the same stress protocol. In this study however, cells were labelled one day before the first stressor. The same may be true for Dajte et al (2009) where chronic stress also did not change the number of BrdU-labelled cells. In this study, BrdU-labelling occurred 4 days before the start of the stress regimen. However, this study did find effects of chronic footshock stress on DCX-positive cells, representing immature neurons, something that was also found by our current study and others using either chronic stress or chronic corticosterone-administration protocols (Nacher et al., 2004; Mayer et al., 2006; Holderbach et al., 2007; Ferragud et al., 2010).

In chapter 5, short-term (4 day) treatment with the GR-antagonist mifepristone normalized the stress-induced reduction in doublecortin and BrdU cell numbers. This observation implies that (1) either mifepristone prevents the reduction of neurogenesis or (2) mifepristone induces a “catch up” of BrdU positive and doublecortin positive cell population by rapidly increasing proliferation or (in case of doublecortin) speeding up neuronal differentiation (i.e. more type 2 cells turn into type 3 cells committed to neural lineage) shortly after the onset of mifepristone application. Regarding option (1), mifepristone could either prevent apoptosis, or stop a decline in proliferation that might have been induced by chronic stress. This implies that the inhibitory effects occur only during the last 4 days of the stress regimen, since a complete normalization occurs (obviously, mifepristone cannot rescue cells that died before the start of treatment).

In order to investigate this in more detail, we recently conducted a new experiment in our lab, in which rats were subjected to 21 days of chronic corticosterone treatment. This paradigm, in combination with four-day mifepristone treatment, was shown to induce similar effects at the level of neurogenesis (Mayer et al, 2006) as did the chronic stress protocol from chapter 5. Preliminary results from this experiment show that the idea of a “catch-up” in proliferation is likely to occur, since 17 days of chronic corticosterone treatment had already significantly reduced the numbers of BrdU and DCX-positive cells. Subsequent treatment for 4 days with mifepristone was again able to rescue this reduction (Hu et al, 2010 in preparation). Interestingly, only one day of mifepristone treatment on day 18 of the corticosterone regimen was already
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sufficient to normalize the reduction in neurogenesis, similar to 4 days of application. The idea that this must be achieved through an increase in proliferation is further confirmed by detailed analysis of the doublecortin cell population. Mifepristone is only able to rescue a subpopulation of doublecortin positive cells; the cells still capable of proliferation (i.e. morphological type A through D, Plumpe et al, 2006), but not the more mature types (E and F).

The underlying mechanism via which chronic stress reduces the number of proliferating cells, is that chronic stress may induce cell cycle arrest by inhibiting cell cycle-related proteins (Heine et al., 2004a; Eisch and Mandymam, 2007). Blocking the effects of stress through GR-blockade may kick-start or disinhibit the population of cells halted in their proliferation (chapter 5). In addition, although apoptosis was increased after acute stress, it was decreased after chronic stress (Heine et al, 2004b). Given this parallel reduction in proliferation and apoptosis under chronic stress conditions, an overall slowing of cell turnover in the dentate gyrus may occur.

4. Functional implications of early life stress-induced changes

We showed that a severely adverse early life event like MD affects both male and female offspring at the level of dentate gyrus structure, which may impair cognitive processing and through this, contribute to the enhanced vulnerability to stress related disorders (Lucassen et al., 2009; Lucassen et al., 2010). However, we found that early life stress does not always impair overall hippocampal function in adulthood as measured by dentate gyrus LTP and hippocampus dependent learning tasks. Here I will discuss the functional consequences of early maternal deprivation.

*Is hippocampus dependent learning affected by stress-induced changes in structure?*

It is not possible to answer this question conclusively, and our data suggest that changes in dentate gyrus structure we observed are probably not the only factors that account for functional differences. However, it is likely that the structure of the hippocampus influences its function and I will try to link some of our findings below.

At the level of the dentate gyrus network, results from chapters 3 and 4 show that synaptic plasticity under normal conditions is not affected by maternal deprivation, independent of sex. The evoked post-synaptic potential, as measured during LTP recordings, is the product of the local dentate gyrus circuitry and does not necessarily represent the output of the total dentate gyrus under physiological circumstances. Therefore, the stress-induced reduction in total granule neurons in females may not be revealed by this functional read-out. On the other hand, altered morphology of individual neurons in males and females or lower levels of neurogenesis after early life stress, as was found in males, are expected to change the excitability of the local network.
Adult born neurons have an enhanced synaptic plasticity (Schmidt-Hieber et al., 2004), and changes in the dendritic tree may also influence individual excitability (Mainen and Sejnowski, 1996; van Ooyen et al., 2002). Concerning neurogenesis, the presence of the GABAergic antagonist bicuculline could have obscured the effect of a reduction in neurogenesis, since newborn neurons are relative insensitive to GABAergic inhibition and the contribution of young neurons to LTP may depend on this particular characteristic (Wang et al., 2000). Also, we measured LTP in the rostral part of the dentate gyrus whereas effects of early stress on neurogenesis were most pronounced in the caudal part of the dentate gyrus. Regarding the dendritic morphology in males, we found a reduction in the number of primary dendrites and a subsequent reduction in the dendritic mass of the proximal part of the dendritic tree, whereas the maximum length of primary dendrites was increased. Since axons from the medial perforant path terminate on this proximal part and an effect on LTP could have been expected. However, the degree of LTP in our current, in vitro study is only in the order of magnitude of ~20% and therefore small differences in excitability due to relative mild structural changes will be difficult to measure. In addition, an increased spine density (both in the proximal and distal part) was measured in MD males, which could have normalized this effect.

In conclusion, in spite of the observed structural changes, dentate gyrus LTP is not affected by early maternal deprivation. Other studies in the context of early life environment did observe differences in LTP on some occasion. For example, chronic early life stress and low levels of maternal care impair LTP in the CA3 (Brunson et al., 2005), dentate gyrus (Bagot et al., 2009) and the CA1 region (Champagne et al., 2008), although in these studies no bicuculline was applied. In addition, MD was shown to cause an impairment of CA1 LTP in vivo (Ryan et al., 2009) and swim stress induced late-phase LTP reinforcement in the dentate gyrus (Gruss et al., 2008).

At the behavioural level, it should first be mentioned that MD did not affect stress-induced corticosterone levels, nor did it change basal and stress-induced anxiety as measured in the elevated plus maze and during fear conditioning, respectively. Effects of maternal deprivation on behaviour were most pronounced in males. First, MD males show an impaired acquisition of the Morris Water without changes in their long term memory. Since neurogenesis has been implicated in the acquisition phase of spatial learning in particular (Gould et al., 1999; Kempermann and Gage, 2002; Clelland et al., 2009), reduced proliferation could be causally involved in this impairment (see Addendum). Next to the observation of an impaired spatial acquisition, it was surprising to find an improvement in both contextual and cued fear conditioning. Contextual fear conditioning is dependent on the amygdala and hippocampus (Phillips and LeDoux, 1992) and a lower number of granule cells (females) or levels of neurogenesis (males) thereby reducing the capacity of the dentate gyrus to process incoming information, could be expected to impair fear memory formation. In females, no difference in the freezing response to the
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context was found, indicating that the reduction in granule neurons does not affect this form of memory. In males, the reduced level of cell proliferation in males might actually explain the improvement in contextual fear conditioning since it was shown that neurogenesis can interfere with fear memory. First of all, fear learning was shown to transiently inhibit cell proliferation (Pham et al., 2005) and increased levels of proliferation and neurogenesis hamper fear memory retrieval (Feng et al., 2001; Farioli-Vecchioli et al., 2009). Recently, it has been suggested that decreased neurogenesis is accompanied by an enhanced retention of LTP in the dentate gyrus and a prolonged hippocampus-dependent retention of fear memory (Kitamura et al., 2009). Together these findings suggest that lower levels of neurogenesis (proliferation) facilitate hippocampus-dependent retrieval of contextual memory, although this does not explain the improvement in cued memory formation.

Is hippocampus dependent learning changed by altered stress responsivity?

Alternatively, or in addition to an explanation based on structural changes, the improved processing of fearful information may be due to increased corticosterone levels (or an increased responsiveness to corticosterone) in animals with an early life stress background. This is supported by the finding that long term potentiation in the presence of the stress hormone corticosterone is improved in MD males (chapter 4), similar to animals with a history of low maternal care (Champagne et al., 2008; Bagot et al., 2009). This indicates altered processing of input in a high stress context, which may be due to changes at the level of the HPA-axis such as an altered MR/GR ratio (De Kloet et al., 1998) or changed CRH levels (Brunson et al., 2001; Heim and Nemeroff, 2001; Plotsky et al., 2005). In male offspring, 24h MD on PND 3 was shown to reduce MR and GR expression, whereas in females GR binding was increased (Sutanto et al., 1996). It should be mentioned though that this is strongly dependent on the exact MD-protocol, since an additional sham injection during PND 3 (as was performed in our studies) abolished the inhibitory effects of MD in males. Other studies using maternal separation or deprivation have shown that MR expression was downregulated in females (Aisa et al., 2007). Comparing animals subjected to low levels of maternal care with high care offspring also showed a downregulation of GR and MR levels (Liu et al., 1997; Champagne et al., 2008).

Interestingly, a study exposing pups to neonatal isolation found an increased synaptic plasticity in the projections from the basolateral amygdala (BLA) to the dentate gyrus after TBS stimulation the BLA and recording from the dentate gyrus in vivo, indicating that emotional ‘tagging’ of memories by the amygdala is enhanced in animals with an early life background (Blaise et al., 2008). Whether this originates from an altered excitability of amygdalar neurons or an increased connectivity between the two regions remains to be shown.

In conclusion, our results indicate that especially in males, early life adversity can -through structural and/ or changes in stress-induced excitability- facilitate
stress related learning and memory function. In addition, the reduced capacity of the dentate gyrus (e.g., reduced neurogenesis, granule cell numbers) may reduce the processing of complex and less relevant information, increasing the signal to noise ratio for relevant i.e. threatening experiences only. In view of this, I propose that early life stress (at least in males), through a combination of changes in stress responsivity and structural plasticity, lowers the stress detection threshold, in other words can enhance the “stress radar”. This allows these animals to better identify and remember potential threats in the environment and thereby increase its chance of survival.

5. Programming aspects of the early life environment

As can be concluded from this thesis, the early environment can program the adult neural and behavioral phenotype and early life stress can be both adaptive and maladaptive. We, and others, have shown that the factors critical to the programming effects of early life stress are sex, severity and timing of the stressor, the presence of compensatory mechanisms and of course the genetic background of the animal. However, whether this renders an individual vulnerable or resilient is also dependent on the later environmental context.

Recently, there has been an increasing appreciation of the role of developmental plasticity; the ability of an individual to adjust its development in order to match environmental circumstances (Gluckman et al., 2007). This has been specifically mentioned in the context of an increased risk of metabolic and heart disease in infants with a reduced birth weight (Barker et al., 1993). Reduced birth weight is accompanied by a lowered metabolism through insulin resistance a.o.. Unfavorable developmental circumstances create a thrifty individual that is adapted to meet an adverse environment (in terms of nutrition) later in life. However, when placed in an environment with abundant nutritional resources, a mismatch is created, which subsequently increases the risk for metabolic and heart disease. This is referred to as the “mismatch hypothesis” (Gluckman et al, 2007). In other words, a mismatch between the perinatal and adult environment leads to suboptimal adaptation. From an evolutionary point of view, adaptive capacity that allows one to match to the expected environment increases fitness, may be especially relevant in the context of stress (Champagne et al., 2009). Animals with a greater ability to recognize and remember threats (like our MD males), are likely to enhance their chance of survival in an adverse environment. However, when this adverse environment is not met, being more stress-responsive may simply be a disadvantage (figure 2). An important question is therefore, when does adaptation change into vulnerability?

As mentioned in the previous section, early maternal deprivation negatively affects structural parameters, impairs spatial learning but also results in an improved stress radar, indicating that in male offspring, predictive adaptation occurs. This is beneficial when the adult environment is matched in
terms of adversity, allowing animals to respond more apt to stress and enhance its chances of survival upon immediate stress. However, in the long run, it is likely that at the level of allostasis a trade off occurs. Cumulative glucocorticoid exposure may lead to toxic effects as was proposed by the glucocorticoid cascade hypothesis (Sapolsky et al., 1986), and may lead to other progressive changes such as an altered MR/GR balance (De Kloet et al., 1998). Therefore, predictive adaptation after early life stress leads to a suboptimal adaptation in a non-threatening environment since it impairs certain aspects of cognitive functioning (as was observed during spatial acquisition) and could make animals more vulnerable after non life-threatening stressors, since stress-induced pathological processes may be amplified in these animals. It seems that adaptive programming does not occur (to the same extend) in female offspring. It could be that in females, this predictive adaptation is not selected for throughout evolution or that it occurs on another level. However, our data is in line with the observation that depressed women show a reduction in their hippocampal volume, since in one of the subfields of the hippocampus a lower number of primary neurons is observed, representing an irreversible change in hippocampal structure. And although we did not find indications of a changed cognitive performance, it is not unlikely that it does lead to a decline in hippocampal function. It is therefore interesting to speculate whether this permanent structural change in females contributes to an increased vulnerability to stress perhaps through an impaired cognitive coping with stressful situations.

6. Concluding remarks and future directions
This thesis was aimed to elucidate how stress shapes the brain, how this can affect structural parameters and function and whether these effects can be normalized by blocking the effects of stress. We found that early life adversity leads to impaired hippocampus structure (chapters 2, 3 and 4), impaired
cognitive function (chapter 4) but an enhanced stress-related functioning (chapters 3 and 4). In particular, we found that males are differently affected than females. Females show rather irreversible effects on structure i.e. a reduced neuron number in the dentate gyrus of the hippocampus, whereas males are impaired at the level of adult neurogenesis. In addition we found that in adult males, the effects of chronic stress on hippocampal neurogenesis can be normalized by the application of a GR antagonist (chapter 5). In terms of function, we found that males are impaired after early life stress in a spatial learning paradigm, while they show enhanced potentiation of the dentate gyrus network in the presence of the stress hormone and enhanced memory formation of a fearful event (chapter 4). We speculate that this may be indicative of predictive adaptation due to early environmental programming, facilitating the processing and storage of stress-related memories.

Whether early life stress indeed leads to adaptive programming resulting in an enhanced ‘stress radar’ should be systematically tested. The hypothesis predicts that when MD males are exposed to a increasingly stressful experience, they would initially (in a non-stress context) show a weaker memory-trace, but upon turning into an adverse event remember this better. Ideally, this should be tested in the same task. In addition, in animals with a history of early life stress an enhanced stress radar may ultimately result in a trade off by accelerating a progressive disruption in homeostasis. This could be tested by subjecting these animals to chronic, unpredictable stress in adulthood which may impact them sooner or to a greater extent.

The data from this thesis do not yet provide a complete picture of the MD-model and several neurotransmitter systems and brain regions are interesting candidates for further research. Of particular interest would be to examine potential changes in structure and function of the amygdala, since we observed an improved cue-dependent fear conditioning. Considering its role in ‘tagging’ emotional experiences through which it promotes memory formation in the hippocampus (Richter-Levin and Akirav, 2003) there is a good possibility that it is affected by early life programming in this model.

Regarding the male/ female differences, an interesting next step would be to test whether the differences are indeed the consequence of differential testosterone levels; in other words, does testosterone facilitate predictive adaptation? Furthermore, our studies do not answer the question whether or how females are functionally affected and we did not observe any major effects in terms of behaviour. However, a smaller dentate gyrus does suggest a lasting and possible irreversible impact of early life stress. Indeed, in males there is still some ‘regenerative capacity’ since lower levels of neurogenesis can be stimulated in the right environment. For example, it was shown that enrichment can normalize behavioural impairments after early life or juvenile stress in male rats (Cui et al., 2006; Ilin and Richter-Levin, 2009). Since MD induces irreversible changes in females and, as mentioned in the previous section, this may lead to an impaired cognitive coping with stressful situations, the effects of a double-hit i.e. adult stressor in female MD offspring may be of particular interest especially
in terms of anxiety and emotional behaviour and in relation to epidemiological literature indicating that females have a higher chance to develop stress-related pathologies like depression when exposed to trauma or severe stress earlier in life (Heim et al., 2002).

In view of stress as a risk factor for depression, and possible strategies to treat stress-related disorders, our studies suggest that a different approach should be chosen in men and women. Here, we show that blocking the effects of stress in adult males can restore structural plasticity, suggesting that translating this to the human situation- men suffering from depression may still benefit from treatment in adult life. This may also be the case after early life stress, since in male rats, the changes in structural plasticity still have the potential to be restored in adulthood. In women however, it may be worth focusing on intervention at an earlier stage in development, since the effects of early life programming during a critical period of development resulted -already in early adulthood- in irreversible changes at the level of brain structure. In conclusion, this thesis supports the shaping effects of stress on the brain but the extent of this effect is highly dependent on the type of stressor, the timing and the sex of the subject. Also, a high-impact stressor like early life stress may prove to be adaptive in adult life, depending on later circumstances. And finally, counteracting the effects of stress results in a rapid normalization of structural plasticity, which offers a promising opportunity in the research of stress-related pathologies.
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