Chronic stress during the peripartum period:

Implications for mother and offspring

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vorgelegt von Katharina Hillerer

aus Passau

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Prologue

"At first I thought what I was feeling was just exhaustion, but with it came an overriding sense of panic that I had never felt before. Rowan kept crying, and I began to dread the moment when Chris would bring her back to me. I started to experience a sick sensation in my stomach; it was if a vise were tightening around my chest. Instead of the nervous anxiety that often accompanied panic, a feeling of devastation overcame me. I hardly moved. Sitting on my bed, I let out a deep, slow, guttural wail. It wasn`t simply emotional or weepy, like I had been told I might be. This was something quite different. This was sadness of a shocking different magnitude. It felt as if it would never go away."

Brook Shields "Down Came the Rain"

What the American actress Brooke Shields describes here in her book *"Down Came the Rain"*, is what about 10-22% of mothers experience after the birth of their child- postpartum depression.

Although, the peripartum period is a time of reduced stress responsivity and increased calmness, for a large percentage of mothers it can represent a time of increased susceptibility to mood disorders. Although, the underlying aetiology of peripartum-associated mood and anxiety disorders is poorly understood, stress exposure during the peripartum period has been shown to be one important risk factor for their development.

Interestingly, both stress and lactation have been associated with alterations in neurogenesis, and this in turn has been shown to be implicated in mood and anxiety disorders.

Therefore, the major aim of my thesis was to assess the effect of chronic peripartum stress on common neuroendocrine and behavioural adaptations and on adult hippocampal neurogenesis. Furthermore, I was interested to determine the effects of early life stress on the behavioural outcome of the offspring in adulthood in dependence on the effects stress exposure had on the mother.

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Chapter 1

General Introduction

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1. Peripartum-related adaptations and psychiatric disorders

Throughout all mammalian species the peripartum period represents a time when a host of changes occur in order to prepare the female for the challenges of motherhood (Walker et al., 1995; Neumann, 2001; Russell et al., 2001). Beside the onset of lactogenesis, milk ejection and maternal behaviours, such as maternal care and aggression, there are numerous other alterations at physiological, cellular and molecular levels that act in concert to ensure the healthy survival and nurturance of the offspring (Brunton et al., 2008; Slattery and Neumann, 2008) (see also summary of findings in *Table 1*). Moreover, there is growing evidence that these changes are required for maternal mental health (Slattery and Neumann, 2008). Thus, the peripartum period represents a time of high risk for women to develop mood and anxiety disorders (Robertson et al., 2004; Beck, 2006; Lonstein, 2007; Bridges, 2008). Here, I describe common peripartum-related adaptations followed by the four most common postpartum psychiatric disorders.

1.1 Neuroendocrine and molecular adaptations in the peripartum period

Towards the end of pregnancy, and into lactation, the response of the hypothalamic-pituitary-adrenal (HPA) axis to a variety of stressors is severely attenuated in mothers (Altemus et al., 1995; Heinrichs et al., 2001). In spite of this, circulating basal glucocorticoid levels are elevated in lactating dams compared with the respective levels in nulliparous females / virgins (Kammerer et al., 2002). Such basal hypercortisolism and concomitant attenuation of HPA-axis response to stress has been identified not only in humans, but also

in numerous other species including rats, mice and sheep (Stern et al., 1973; Barlow et al., 1975; Keller-Wood, 1995; Neumann et al., 1998b; Johnstone et al., 2000; Neumann et al., 2000a; Lightman et al., 2001). The heightened basal activity of the HPA axis in lactation might be due, at least in part, to an increased expression of the neuropeptide vasopressin (AVP) in parvocellular paraventricular nucleus (PVN) neurons of the hypothalamus (Walker et al., 2001b), accompanied by an enhanced sensitivity of the pituitary to AVP (Toufexis et al., 1999). In turn, animal studies have shown that the reduced peak in HPA axis activity predominantly results from a markedly reduced corticotropin-releasing hormone (CRH) mRNA expression in the PVN (Windle et al., 1997; Shanks et al., 1999; Walker et al., 2001b), as well as reduced CRH binding in the adenohypophysis during pregnancy (Neumann et al., 1998b) and lactation (Toufexis et al., 1999). Taken together, these alterations lead to reduced CRH production and release by PVN neurons, which is also accompanied by a decreased secretory response of the adrenocorticotropic hormone (ACTH) to CRH (Neumann et al., 1998a; Toufexis et al., 1999). Further central changes, which occur to attenuate the HPA axis responsiveness, include an altered pattern of the excitatory inputs to the hypothalamus (Douglas et al., 1998). During the peripartum period, a decreased noradrenergic tone within the PVN and a reversed opiodergic system inhibit the HPA axis activity around birth (Douglas et al., 1998; Toufexis et al., 1998; Wigger et al., 1999; Kammerer et al., 2002). In parallel with the reduction of these excitatory inputs to the hypothalamus, inhibitory pathways, including those of oxytocin (OXT) and prolactin (PRL), are highly activated. These two neuropeptide systems mediate key roles in reproductive functions, such as the promotion of labour, lactogenesis, milk ejection and maternal behaviour (Pedersen and Prange, 1985; Bridges, 2008; Bosch, 2011), but moreover, modulate the HPA axis response to stress (Neumann et al., 2000a; Torner et al., 2002). The

oxytocinergic system within the hypothalamus undergoes fundamental structural and functional reorganization in the peripartum period. This is reflected by altered size and branching patterns of dendritic trees within the PVN and supraoptic nucleus (SON) (Stern and Armstrong, 1998) and increased synaptic and neuronal-glial interaction (Theodosis and Poulain, 1993; Hatton, 1997). Further, OXT mRNA, OXT receptor (OXT-R) expression in the PVN (Zingg et al., 1995; Figueira et al., 2008) and central OXT release (Neumann et al., 1993; Neumann et al., 2000a; Neumann et al., 2000b) are increased in the peripartum period. The changes in the OXT system are accompanied by an upregulation of the PRL system. PRL receptors (PRL-Rs) in the PVN, SON and the medial preoptic area are increased during the peripartum period (Grattan et al., 2001; Kokay et al., 2006), which plays an important role in the parallel reduction in stress-responsiveness (Torner et al., 2001; Torner et al., 2002). Given the similarity in the actions of OXT and PRL, it is not surprising that there is substantial evidence for an interaction between these two neuropeptides. Thus, PRL-Rs have been described on OXT (and AVP) magnocellular PVN neurons (Grattan et al., 2001; Kokay et al., 2006) and PRL administration has been shown to increase OXT release and OXT mRNA *in vitro* (Ghosh and Sladek, 1995b, a). On the other hand, OXT administration can increase PRL release from the pituitary of oestrogen-primed ovariectomised rats (Samson et al., 1986). These effects may be mediated *via* a PRL-induced increase in nitric oxidase synthase activity in pregnant rats and in males (Popeski et al., 2003; Vega et al., 2010). However, as recently demonstrated, cells that increase in PRL sensitivity, as assessed five weeks after weaning, are non-oxytocinergic neurons suggesting that the upregulation observed in the peripartum period may be in CRH or AVP neurons (Sjoeholm et al., 2011). Taken together, the majority of available evidence suggests that the OXT and PRL system act synergistically in the peripartum period, to protect the late pregnant and lactating mother from over-responding

to a stressor. Moreover, such alterations are likely in turn to alter the behavioural repertoire shown during this time.

Interestingly, beside those alterations in the PVN, there are also changes at the level of the hippocampus that occur during the peripartum period, including hippocampal neurogenesis (Leuner et al., 2007). However, since this is the focus of Section 3 it will be discussed in more detail there.

1.2 Behavioural adaptations during the peripartum period

Obviously, one key behavioural change that occurs in the peripartum period is the display of maternal behaviour, including maternal aggression, as most mammalian species do not display spontaneous maternal behaviour. Brain factors that significantly contribute to the onset and maintenance of the complex repertoire of maternal behaviours include the elevated availability of the neuropeptides OXT (van Leengoed et al., 1987; Russell et al., 2001), PRL (Bridges et al., 1997; Torner et al., 2002), as well as AVP (Bosch and Neumann, 2010; Bosch et al., 2010; Bosch, 2011) and their receptors (Insel, 1990; Zingg et al., 1995; Pi and Grattan, 1999; Figueira et al., 2008; Caughey et al., 2011). Alterations in these hormonal systems act in concert to result in decreased anxiety during lactation (Neumann et al., 2000a; Walker et al., 2001b; Torner et al., 2002; Larsen and Grattan, 2010). In detail, treatment with an OXT antagonist 10 min prior to elevated plus- maze (EPM) testing increased anxiety-related behaviour in pregnant and lactating, but not virgin rats, suggesting that the anxiolytic action of endogenous OXT is restricted to times when its activity is elevated, such as the peripartum period (Neumann et al., 2000a). Chronic administration of

a PRL-R antisense oligonucleotide increases anxiety-related behaviour in lactating dams (Torner et al., 2002), while administration of bromocriptine, an agent that reduces PRL secretion (Popeski et al., 2003), during early pregnancy leads to elevated levels of anxiety in the postpartum period (Larsen and Grattan, 2010). These findings suggest that the peripartum-associated increase in PRL and OXT system activity is of vital importance for the decrease in anxiety during lactation. Moreover, the relationship between decreased anxiety and increased aggression during the lactation period has been shown in rodents (Maestripieri and D'Amato, 1991; Parmigiani et al., 1999; Bosch et al., 2005).

Both, the rapid fall in plasma progesterone and testosterone (that occurs between pregnancy day (PD)18 and 21 in rodents), as well as the decreased activity of the CRH system, are implicated in the onset of maternal behaviour and aggression (Pedersen and Prange, 1985; Rosenblatt et al., 1988; Albert et al., 1992). Thus, increasing CRH availability, either by intracerebroventricular (icv) injection of CRH, or genetically knocking out CRH binding protein, results in lower levels of maternal aggression in mice (Gammie et al., 2004; Gammie et al., 2008). These findings suggest that the peripartum-associated decrease in CRH system activity is important for the expression of maternal aggression and reduced anxiety in addition to the aforementioned role in reduced stress responsivity. Both increased maternal offensive behaviour, as well as a reduced anxiety are important adaptations to ensure the protection of the offspring (Erskine et al., 1978; Hard and Hansen, 1985). However, it is also likely that this peripartum-associated anxiolysis, which is also observed as an increased calmness particularly in breast-feeding mothers (Carter et al., 2001; Heinrichs et al., 2001; Bridges, 2008), is important for maternal mental health.

Table 1: Examples of neuroendocrine and behavioural alterations observed in pregnancy and lactation assessed in rodent and human studies

Common peripartum adaptations	Time of occurrence	References animal studies	References human studies
Neuronal and neuroendocrine adaptations			
Chronic basal hypercorticism/hypercortisolism	lactation	(Stern et al., 1973; Walker et al., 1995; Windle et al., 1997; Lightman et al., 2001)	(Altemus et al., 1995; Kammerer et al., 2002; de Weerth and Buitelaar, 2005; Taylor et al., 2009)
Deceased responsiveness of HPA axis to stressors	lactation	(Stern et al., 1973; Windle et al., 1997; Neumann et al., 1998b; Shanks et al., 1999; Brunton and Russell, 2003; Douglas et al., 2003)	(Heinrichs et al., 2001; Heinrichs et al., 2002; Meinlschmidt et al., 2010)
Decreased CRH mRNA expression in the PVN	lactation	(Windle et al., 1997; Neumann et al., 1998b; Tilbrook and Clarke, 2006)	n.a.
Decreased CRH binding in the adenohypophysis	pregnancy and lactation	(Neumann et al., 1998a; Toufexis et al., 1999; Tilbrook and Clarke, 2006)	n.a.
Decreased noradrenergic input to PVN neurons	end of pregnancy and lactation	(Toufexis et al., 1998; Douglas et al., 2005)	n.a.
Attenuated pituitary sensitivity to CRH	pregnancy and lactation	(Neumann et al., 1998b; Toufexis et al., 1999)	n.a.
Altered size and branching pattern of dendritic trees in the PVN	lactation	(Stern and Armstrong, 1998)	n.a
Altered synaptic neuronal-glial interaction	lactation	(Theodosis, 1993; Hatton, 1997)	n.a
Increased OXT mRNA and OXT-R mRNA expression in the PVN	lactation	(Insel, 1990; Zingg et al., 1995; Windle et al., 1997; Bosch and Neumann, 2008; Figueira et al., 2008; Slattery and Neumann, 2008)	n.a
Decreased ACTH secretion by systemic OXT after stress	lactation	(Legros, 2001)	n.a.
Behavioural alterations			
Increased maternal behaviour	lactation	(Pedersen and Prange, 1985; Rosenblatt et al., 1988; Neumann, 2001; Russell et al., 2001)	(Rosenblatt, 1994; Russell et al., 2001)
Increased maternal aggression	lactation	(Erskine et al., 1978; Neumann, 2001; Bosch and Neumann, 2010; Caughey et al., 2011)	n.a.
Increased calmness, decreased anxiety and responsiveness to stressors	lactation	(Neumann et al., 2000b; Carter et al., 2001; Torner et al., 2002)	(Altemus et al., 1995; Heinrichs et al., 2001)

1.3 Postpartum mood and anxiety disorders

Despite the adaptations outlined above, the peripartum period represents the time of highest vulnerability for women to develop psychiatric illness, including mood and anxiety disorders (Hillerer et al., 2011a). Typically, postpartum mood and anxiety disorders are divided in four main groups:

Postpartum blues (PPB; aka *Baby blues):* This short-lasting mood disturbance occurs between the 3rd and 5th day postpartum and is believed to be a normal reaction to changing steroid-hormone levels. Thus, oestradiol levels increase 50-fold and progesterone levels 10-fold throughout pregnancy but drop to early follicular levels within 1 week after delivery (Tulchinsky et al., 1972; Poindexter et al., 1983). About 30-75 % of mothers are affected (Stein, 1981) and exhibit symptoms like mood lability, frequent crying, sleep disturbance, irritability and anxiety (Pitt, 1973; Stein, 1981).

Postpartum Depression (PPD): This major depressive disorder, which starts within the first four weeks after birth, occurs in 10-22 % of mothers and can be a result of long-lasting PPB (O'Hara et al., 1984; Josefsson et al., 2001). The symptomatology is indistinguishable from depressive episodes occurring at other times during a woman's life (Cooper et al., 1988), with core symptoms being emotional lability, mental confusion and anxiety / insecurity (Beck and Gable, 2002; Beck and Indman, 2005). Moreover, a loss of interest in the baby, aversive reactions to infant stimuli and crying, as well as impaired or even lack of maternal care are observed in postpartum depressed women (Lyons-Ruth et al., 1986; Bifulco et al., 2004).

Postpartum anxiety: Often coincident with PPD, postpartum anxiety disorder has an incidence of 5-12% and generally occurs in the first six month after birth (Heron et al., 2004). There is even some evidence that the rate of anxiety disorders peripartum is higher than the

rate for depression (Andersson et al., 2006). The symptoms of postpartum anxiety often overlap with those of PPD, which makes them hard to distinguish. However, a highly protective maternal style, termed “helicopter parenting” seems to be a prominent feature of postpartum anxiety that distinguishes it from PPD (Barnett and Parker, 1986; Bridges, 2008).

Postpartum Psychosis (PPP): As arguably the most serious postpartum mood disorder, PPP appears within the first two weeks after delivery (Klompenhouwer et al., 1995) and has an incidence of 1 to 2 per 1000 mothers (Kendell et al., 1987; Kumar, 1994). These mothers often show bipolar symptoms (i.e. euphoria, over-activity, irritability, violence) (Kendell et al., 1987; Heron et al., 2005), or have severe depression with delusions or verbal hallucinations (Wisner et al., 1994). Some of these mothers switch from mania to depression (or *vice versa*) within the same episode. PPP always requires the sufferers to be hospitalised, because of the danger of suicide and infanticide, which is a vital difference to PPD.

Although the symptomatology of postpartum mood and anxiety disorders is well known, the mechanisms underlying the aetiology remain elusive. In contrast, various risk factors have been identified that increase their likelihood, including a prior history of anxiety or depression experienced during pregnancy, a low socioeconomic status, smoking or alcohol abuse (Heron et al., 2004; Homish et al., 2004; Beck, 2006; Hall and Holden, 2008; Friedman and Resnick, 2009; Soderquist et al., 2009). Importantly, one translational risk factor for the development of these disorders is chronic stress during pregnancy (Robertson et al., 2004).

2. Consequences of chronic stress on peripartum-related adaptations

Given the clear association between stress and mood / anxiety disorders (Cryan and Slattery, 2007), including postpartum disorders (see above), assessing the impact of stress on peripartum-associated adaptations might help to identify potential mechanism involved in the course of those disorders. The following section describes the findings from basic research on the systems described above that are known to be altered during the peripartum period and / or from human studies assessing postpartum mood and anxiety disorders.

2.1 Stress-effects on neuroendocrine adaptations

A common finding observed in patients with psychiatric disorders is dysregulation of the HPA axis (Plotsky et al., 1998; Holsboer, 2001; Gillespie and Nemeroff, 2005), which is also seen in mothers with postpartum mood and anxiety disorders (Brunton et al., 2008; Slattery and Neumann, 2008; Brummelte and Galea, 2010b). However, the results are partially controversial. There are human studies demonstrating a decreased awakening cortisol level in women with postpartum depression (Taylor et al., 2009), whereas other studies in women with postpartum thoughts of harming the infant, revealing an increase in ACTH levels 48h postpartum, with no differences in CRH and cortisol levels (Labad et al., 2011). Although the changes in HPA axis activity seem to depend on the type of postpartum mood disorder, increased ACTH levels and hypocortisolism may represent appropriate biomarkers for postpartum psychopathologies. Available data from basic research further indicate that

alterations in HPA axis (re)activity may play an important role in postpartum mood and anxiety disorders. Rats subjected to repeated strobe-light stress throughout pregnancy and lactation show an enhanced corticosterone (CORT) response to stress in the afternoon compared to the morning (Leonhardt et al., 2007). Moreover, studies in primates show that daily injection of hydrocortisone, causing a chronic elevation in cortisol levels, impair mother-infant interactions, as seen in a reduction in infant-carrying and infant-inspection after a noise stressor test (Saltzman and Abbott, 2009). Thus, chronic peripartum stress can affect both the basal hypercorticism / hypercortisolism, as well as the attenuated stress responsivity observed in lactation. Therefore, the findings from human and animal studies suggests that an optimal CORT / cortisol window exists, in which an elevation above nulliparous / virgin levels is beneficial, while the higher levels observed after chronic stress exposure negatively affect maternal mental health and maternal care. Consequently, it is suggested that such stress-induced prevention of these peripartum-associated neuroendocrine adaptations may underlie postpartum mood disorders (Neumann, 2001; Brunton et al., 2008; Slattery and Neumann, 2008), but further studies are required to investigate this link in more depth.

2.2 Stress-effects on maternal behaviour

Numerous studies in rodents using different chronic stress paradigms reveal that chronic stress during pregnancy results in altered maternal behaviour, including maternal care and aggression. Maestripieri and co-workers could demonstrate that mice that received daily 2 h restraint stress (RS) between PD4 and PD14 expressed decreased maternal aggression in the

resident- intruder test and increased latency to retrieve the pups in a novel environment during the pup-retrieval test (Maestripieri and D'Amato, 1991). Similarly, an ultra-mild stress paradigm (cage tilt; confinement in a small cage and paired housing; overnight period with difficult access to food; overnight period with permanent light; overnight period in soiled cage; reversal of light/dark cycle), delivered over 1 h in the morning, 2 h in the afternoon and during night, from the day of mating till parturition, also resulted in an increased latency in the pup retrieval test (Pardon et al., 2000). However, while pup retrieval latency was increased, other components of maternal care were not changed as a result of this stress paradigm (Pardon et al., 2000). Studies in rats exposed either to repeated RS (Smith et al., 2004) or high doses of CORT (40mg/kg) (Brummelte and Galea, 2010a) between PD 10 and 20, show a decrease in kyphotic (arched-back) nursing. Moreover, in different non-human primate species stress-induced increases in plasma cortisol concentrations are associated with abnormal maternal behaviour, like infant neglect (Bahr et al., 1998; Brent et al., 2002). In a recent study Saltzmann and Abbott demonstrated that exogenous (subcutaneous) administration of cortisol to lactating female marmosets impaired maternal motivation and interfered with the expression of appropriate maternal behaviour (Saltzman and Abbott, 2009). Furthermore, in rhesus macaque females that abuse their infants, high levels of aggression are observed, as well as elevated concentrations of CRH in the cerebrospinal fluid (CSF) (Maestripieri et al., 2005). The latter further supports the findings in rodents showing that reversal of the peripartum-associated decrease in CRH leads to an attenuation of the concomitant increase in aggression. Furthermore, acute separation from the pups, also a form of stress, affects maternal behaviour with both a reduced maternal behaviour (Boccia et al., 2007) and a higher intensity of maternal care being observed (Francis and Meaney,

1999). Thus, chronic stress exposure during the peripartum period can lead to decreased maternal care and attachment.

2.3 Stress-effects on maternal anxiety

Interestingly, studies in rodents, non-human primates and humans indicate a correlation between maternal behaviour and anxiety-related behaviour. Mothers with high levels of anxiety express high maternal motivation and highly protective maternal styles (Maestripieri, 1993b, a; Neumann et al., 2005a; Bosch, 2011), known as “helicopter parenting” in humans (Barnett and Parker, 1986; Bridges, 2008). Given the known correlation between maternal behaviour and anxiety-related behaviour, the results of Darnaudery et al. seem to be of special importance, as they reveal that dams exposed to chronic RS (3 x 45 min / day) during the last week of gestation showed increased anxiety-related behaviour on the EPM when tested 26 days post-stress (Darnaudery et al., 2004). Importantly, chronic stress had no effect on anxiety-related behaviour of virgins, pointing out the specificity of chronic stress on peripartum-associated adaptations. Thus, greater understanding of the underlying mechanisms of peripartum-associated anxiety, and the subsequent alterations in these neurobiological mechanisms by chronic stress exposure, may provide better insight into postpartum mood and anxiety disorders.

2.4 Stress-effects on maternal depression-like behaviour

In contrast to the positive correlation between maternal behaviour and anxiety, there appears to be an opposite correlation between maternal behaviour and depression-like behaviour. Thus, mothers that show low levels of maternal care tend to show increased depression-like behaviour. Although, there are only few studies available to date assessing the effect of chronic stress during the peripartum period on depression-like behaviour of the dam, increase in depression-like behaviour, as assessed by forced swim test (FST), has been found throughout different paradigms, using either repeated RS during pregnancy (Smith et al., 2004), acute separation stress during early lactation (Boccia et al., 2007) or repeated administration of CORT between postpartum day 2 and 24 (Brummelte and Galea, 2010a). In the latter, increased depression-like behaviour was further correlated with a decrease in maternal care (Brummelte and Galea, 2010a). As stated above, rodents and non-human primates with naturally or exogenous induced high levels of plasma CORT / cortisol show symptoms similar to those seen in mothers with PPD, e.g. decreased infant attachment and maternal motivation. Therefore, further studies in such models will greatly assist to determine the mechanisms underlying postpartum mood disorders and may thus lead to better treatment options.

2.5 Stress-effects on the maternal OXT system

Given the importance of elevated OXT in the peripartum period, in addition to its stress-attenuating properties, a number of studies have assessed the impact of stress on this neuropeptide in dams. The importance of the OXT system in attachment, maternal

behaviour and its concurrent susceptibility to stress could be shown in recent human and animal studies. Meaney and co-workers revealed that dams showing a high frequency in licking and grooming behaviour (high LG dams) express higher levels of OXT-R in distinct brain regions known for their importance in regulating maternal behaviour, like the bed nucleus of the stria terminalis (BNST), the medial preoptic area and the central amygdala than dams with a low frequency in this behavioural pattern (low LG dams) (Francis et al., 2000). Interestingly, repeated exposure to RS between PD15 and PD21 (3 x 30 min per day) resulted in a decrease in the expression of OXT-R in high LG dams comparable to those of low LG dams with a concurrent decrease in LG behaviour e.g. a reduction in maternal behaviour (Caldji et al., 1998; Francis et al., 2000; Champagne et al., 2001; Champagne and Meaney, 2006). The importance of OXT in maternal attachment and mood regulation is confirmed by several human studies. Thus, low OXT plasma levels are found in first-time mothers who display low infant attachment ratings (Strathearn et al., 2009), as well as in cocaine-addicted mothers, who are more likely to have psychiatric disorders and less attachment to their infants (Light et al., 2004). Moreover, low plasma OXT concentrations in mid-pregnancy significantly predicts the development of postpartum depression at two weeks postpartum (Skrundz et al., 2011). In summary, the findings from rodent studies employing peripartum stress exposure and those from human patients suggest that OXT may also represent a marker for postpartum mood and / or anxiety disorders.

3. Adult hippocampal neurogenesis under physiological conditions

“In the adult centers, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated” (Cajal, 1913). This statement by Santiago Ramon y Cajal, long remained a central dogma of neuroscience and implicates that neurogenesis is restricted to the prenatal and early postnatal period and does not occur in adulthood. About fifty years later this theory began to falter, when Altman and Das provided the first evidence that the production of new neurons in the dentate gyrus (DG) of the hippocampus persists into adulthood (Altman, 1963; Altman and Das, 1965). Until then, there was insufficient evidence, and available techniques, to determine the phenotype of these cells. However, another ten years later, Kaplan and Hinds could successfully identify the newly generated cells as neurons, which become morphologically and functionally identical to mature granule cells (Kaplan and Hinds, 1977). Although neurons were the first newly generated cells identified it is now known that there is also a production of glial cells possible (for further information see below).

To date, adult hippocampal neurogenesis is confirmed in numerous different species, including mice, rats, tree shrews, marmosets, macaques and humans (Cameron et al., 1993b; Gould et al., 1997; Kempermann et al., 1997; Eriksson et al., 1998; Gould et al., 1998; Gould et al., 1999). In the adult mammalian brain, there are two neurogenic regions that are generally accepted, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. Precursor cells that reside in the anterior portion of the SVZ in the walls of the lateral ventricles migrate along the rostral migratory stream (RMS) into the olfactory bulb, where they differentiate into neurons. Although the reports are conflicting, the production of new neurons also seems to occur in the neocortex, the amygdala and the hypothalamus

(Fowler et al., 2002; Dayer et al., 2005). My thesis was focused on the SGZ, given the fact that this neurogenic region is more implicated in stress and affective disorders (for reviews see Santarelli et al., 2003; Balu and Lucki, 2009; Lucassen et al., 2010). Therefore, the further sections refer to neurogenic processes in the SGZ of the hippocampus.

3.1 Cell types and specific markers of hippocampal neurogenesis

The adult hippocampal neurogenesis that occurs in the neurogenic region of the DG, the SGZ, comprises of at least four distinct processes: cell proliferation, differentiation, migration and cell survival (see also *Figure 1*). The SGZ harbors a residing population of cells that are believed to be multipotent nuclear stem cells (NSCs) (Kempermann et al., 2004). NSCs have some characteristics of astroglial cells and as such express the astrocytic marker glial fibrillary acid protein (GFAP) – however, they are not themselves glial cells. They are either in a quiescent stage, in which they are in G_0 of the cell cycle (when they express SOX2, a transcription factor essential for the maintaining self-renewal of stem cells), or self-renewing *via* mitosis (when they express proliferating cell nuclear antigen; PCNA). NSCs that are actively engaged in cycling, give rise to daughter cells (progenitor cells), which act as transiently amplifying cells (McKay, 1997; Seaberg and van der Kooy, 2002, 2003) and express the microtubule-associated protein doublecortin (DCX), a marker for commitment to a neuronal phenotype (Seri et al., 2001; Brown et al., 2003; Seri et al., 2004). These progenitor cells re-enter the cell cycle to divide again for the production of daughter cells that become post-mitotic and differentiate. The majority of these cells (about 70%)(Cameron et al., 1993a) differentiate into neurons (Cameron and McKay, 2001), as they express the

neural nuclei protein NeuN (Kempermann, 2005), whereas only a few (about 10% (Cameron et al., 1993a)) become astroglial cells that express GFAP (Kempermann et al., 1997). After differentiation, cells migrate to the granule cell layer (GCL), where, after approximately seven weeks after division, the new cells are functionally indistinguishable from older cells (Zhao and Overstreet-Wadiche, 2008) and are electrophysiologically integrated into the circuitry (Stanfield and Trice, 1988; Hastings and Gould, 1999; van Praag et al., 2002).

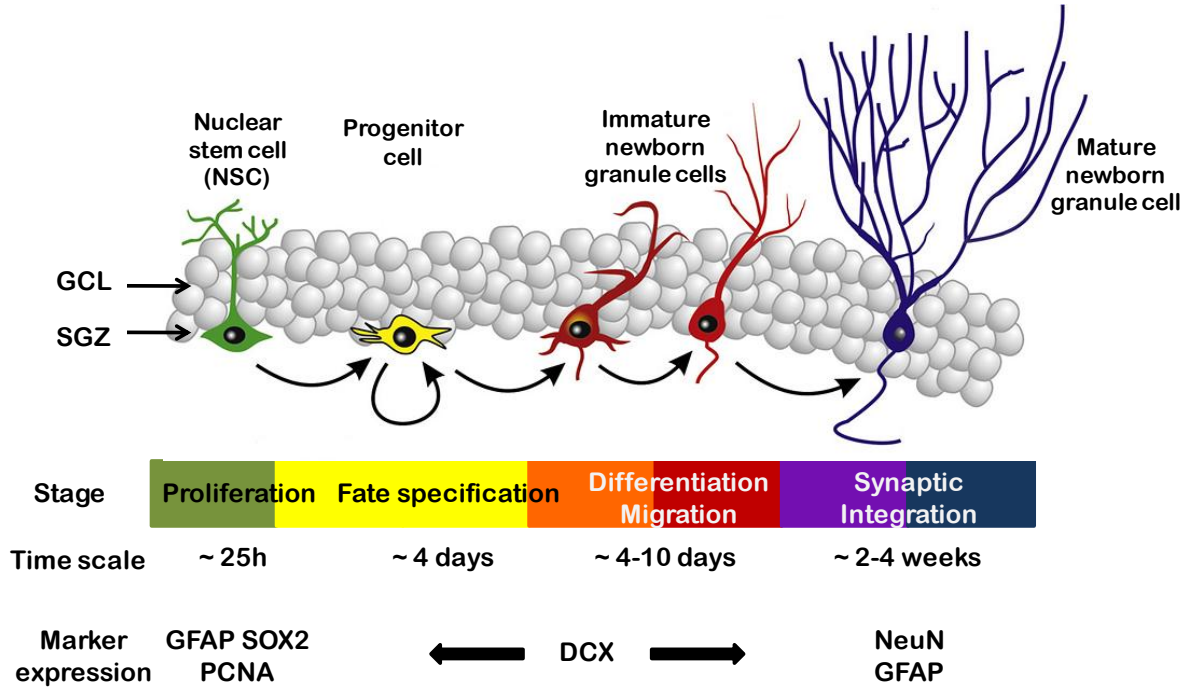

Figure 1: Schematic representation of the different stages of adult hippocampal neurogenesis

NSCs located in the SGZ self-renew *via* mitosis and give rise to progenitor cells. They act as transiently amplifying cells that re-enter the cell cycle to divide again to produce daughter cells that become post mitotic (immature newborn granule cells). By ongoing differentiation processes these cells develop an either neuronal or astroglial phenotype. After the differentiation process, these cells migrate to the GCL where they get functionally integrated in the circuitry (mature newborn granule cell).

3.2 Functional importance of hippocampal neurogenesis

The fact that adult generated neurons get morphologically and functionally integrated in the hippocampal circuitry suggests that they play an important role in several hippocampal-dependent functions by modification and refinement of the existing neuronal circuitry in the hippocampus (Imayoshi et al., 2008). It is well established that adult hippocampal neurogenesis plays an important role in learning and memory and *vice versa* (for reviews see Leuner et al., 2006; Koehl and Abrous, 2011). Thus, rodent studies revealed that treatment with methylazoxymethanol acetate (MAM) or low dose irradiation to impair hippocampal neurogenesis, affects the consolidation of long-term spatial memory and contextual fear memory and impairs trace conditioning (Shors et al., 2001; Snyder et al., 2005; Ko et al., 2009). Moreover, females outperform males in the trace eyeblink conditioning task, and concomitantly retain a greater number of newborn cells (Dalla et al., 2009). Beside this role of SGZ neurogenesis, there is increasing evidence that it is also involved in the pathogenesis of stress-related disorders, such as depression. Magnetic resonance imaging (MRI) studies in humans revealed that depressed patients show a decrease in hippocampal volume (Sheline et al., 1999; Bremner et al., 2000; Campbell et al., 2004), which might be due to increased apoptosis of neurons or decreased neurogenesis in the DG of the hippocampus (Sahay et al., 2007). In contrast, antidepressant treatment increases the number of proliferating progenitor cells in patients with major depressive disorder (MDD) (Boldrini et al., 2009). This correlates well with animal literature showing that not only do antidepressants increase hippocampal neurogenesis (Wang et al., 2008; Couillard-Despres et al., 2009; Dagyte et al., 2011), but that this effect is necessary for their behavioural effects in animal and human studies (Czeh et al., 2001; Santarelli et al., 2003; Snyder et al., 2011). This correlation between neurogenesis, depression and the effect of antidepressants is often referred to as

the “neurogenic theory of depression”. Given the fact that depression is often associated with HPA axis dysregulation (Carroll et al., 1968) and that the hippocampus is the main negative regulatory feedback site of the HPA axis, it is not surprising that adult-generated cells are implicated in a proper inhibitory control of the HPA axis (Schloesser et al., 2009; Snyder et al., 2011). Apart from the effects of adult hippocampal neurogenesis in non-reproductive functions, it also seems to be important during the peripartum period (see below and Leuner and Shors, 2006; Leuner et al., 2007; Furuta and Bridges, 2009). In summary, studies show that continuous plasticity throughout the hippocampal circuitry is essential for the behavioural pattern of the organism.

3.3 Sex differences in hippocampal neurogenesis

Numerous biological processes exhibit profound sexual dimorphism, including the structure and function of the hippocampus. Thus, studies in rats demonstrate that males have a larger GCL (Roof and Havens, 1992; Roof, 1993), more dendritic intersections in DG granule cells (Juraska et al., 1989), as well as a greater number of mossy fiber synapses in the hilus (Madeira and Paula-Barbosa, 1993), when compared with females. In contrast, females display an increased number of synapses in the CA3 region of the DG compared to males (Madeira et al., 1991). With respect to sex differences in adult hippocampal neurogenesis, the results are somewhat controversial throughout the literature. The number of apoptotic cells seems to be higher in females than in males, as the latter express a higher percentage of surviving cells (males: 75%; females 47%), even if their total number is not changed (Tanapat et al., 1999). With regard to the number of granule cells in the DG, the results are

more controversial, with studies showing increased numbers in males compared with females (Madeira and Paula-Barbosa, 1993) as well as the other way round (Handa et al., 1994). Nonetheless, contemplating the literature as a whole, it can be seen that adult hippocampal processes are differentially regulated between males and females.

In addition to the array of structural and cellular differences mentioned above, males and females differ in hippocampal-dependent tasks, with females being better in tasks that involve memory of personal experiences and males being better in tasks that require complex spatial information (de Frias et al., 2006; Shors, 2006b; Dalla et al., 2009). These findings suggest that sexual steroids, and their receptors, could play an important role in the regulation of hippocampal neurogenesis (Tanapat et al., 1999; Galea et al., 2006). As such, oestrogen with its naturally fluctuations across the oestrous cycle positively correlates with cell proliferation in females (Tanapat et al., 1999; Banasr et al., 2001; Tanapat et al., 2005). Accordingly, cell proliferation is increased during proestrus when oestrogen levels are naturally high, whereas lowering oestrogen levels by ovariectomising females, decreases cell proliferation, as well as oestrogen-receptor density in the DG (Tanapat et al., 1999; Rose'Meyer et al., 2003). Despite numerous studies assessing the effects of oestrogen on hippocampal neurogenesis in females, the role of this sex-steroid in males remains largely unknown (Galea et al., 2006). Although, androgens could be involved in cell proliferation and cell survival, especially in males, there have not been any systemic studies assessing the potential regulatory role of such hormones in the DG of mammals (Galea et al., 2006).

3.4 Neurogenesis during the peripartum period

Beside the numerous changes in neuroendocrine and behavioural parameters, as well as hormonal systems (as outlined in Section 1), the peripartum period is also characterised by numerous alterations in hippocampal morphology (Hamilton et al., 1977; Galea et al., 2000; Oatridge et al., 2002; Kinsley et al., 2006; Pawluski and Galea, 2006). Thus, decreased dendritic pruning of CA1 and CA3 pyramidal neurons (Pawluski and Galea, 2006), as well as increased spine density, are observed during pregnancy and lactation (Kinsley et al., 2006). Despite knowledge of these changes, and the fact that hippocampal-dependent tasks, such as spatial memory (Tomizawa et al., 2003; Pawluski et al., 2006), are altered during the peripartum period, there are only a few studies assessing adult hippocampal neurogenesis during this critical and plastic period.

Gould and co-workers were the first to show that cell proliferation and cell survival in the DG are decreased during the lactation period (Leuner et al., 2007); findings which have been subsequently recapitulated by others (Darnaudery et al., 2007; Pawluski and Galea, 2007). In a series of elegant experiments, Leuner et al. additionally revealed that the decreased cell proliferation is dependent on the lactation-associated basal hypercorticism. Thus, removal of the pups immediately after birth, or adrenalectomy, both manipulations which reduce plasma CORT levels, restored cell proliferation in dams to the level of virgins (Leuner et al., 2007). Beside the alteration in glucocorticoid levels during the peripartum period, there are fluctuations of several other hormones that are known for their importance in motherhood, such as PRL, AVP and OXT (as described in Section 1), which are also implicated in the regulation of neurogenesis. Thus, the high levels of PRL during pregnancy stimulate the proliferation of NSCs in the SVZ of mice (Shingo et al., 2003), while PRL administration regulates cell proliferation in the SGZ and embryonic astrocytes in rats and mice (Mangoura

et al., 2000; Torner et al., 2009). To date, there has only been a single study assessing the role of OXT and AVP on hippocampal neurogenesis. This study, performed in male rats, revealed a positive effect of acute and chronic peripheral, as well as local administration of OXT in one hemisphere of the hippocampus on cell proliferation in the DG, even under stressed conditions. Interestingly, acute peripheral injection of AVP had no effect on hippocampal neurogenesis (Leuner et al., 2010). More studies are needed to get a better insight into the functional role of alterations in hippocampal neurogenesis during the peripartum period. Furthermore, studies are required to determine in more detail the consequences of pregnancy and lactation on distinct processes of hippocampal neurogenesis, including neuronal and astroglial differentiation and stem cell quiescence.

4. Consequences of chronic stress on adult hippocampal neurogenesis

Given the link between chronic stress and the development of depression and the fact that neurogenesis seems to be involved in the pathogenesis of stress-related disorders like depression, the following section will elaborate the consequences of stress on adult hippocampal neurogenesis.

As stated above, the hippocampus plays an important role in the stress response by regulating the release of CRH *via* a negative feedback loop to the hypothalamus, and as such is adversely affected by stress exposure (Herman et al., 2008; Reber, 2011). In detail, repetitive, uncontrollable and unpredictable stress leads to profound alterations in all hippocampal subregions, including retraction of pyramidal neuron dendrites, reduction in dendritic spine density and synapse loss in the CA3 region of the hippocampus (Magarinos et al., 1996; McKittrick et al., 2000; Stewart et al., 2005; Chen et al., 2008), as well as a dendritic regression and spine loss in the DG (Sousa et al., 2000; Hajszan et al., 2009). Beside these effects, in several mammalian species stress exposure has generally an anti-neurogenic effect. Both physiological (RS, foot shock) (Malberg and Duman, 2003; Pham et al., 2003) and psychological stressors (subordinate stress, resident intruder stress, isolation stress, predator odor) (Gould et al., 1997; Gould et al., 1998; Tanapat et al., 2001; Falconer and Galea, 2003) affect one or more phases of the neurogenesis process, i.e. proliferation, survival and differentiation. In general, it is assumed that there is a trend towards the more intense and prolonged stressors resulting in more robust and detrimental effects. Although the underlying mechanisms are largely unknown, there is considerable evidence that stress

affects hippocampal neurogenesis in a sex-specific manner (see also *Table 2*). Accordingly, chronic stress decreases cell proliferation in males (Tanapat et al., 2001; Falconer and Galea, 2003; Pham et al., 2003; Heine et al., 2004), whereas this process appears to be unaffected in females (Tanapat et al., 2001; Falconer and Galea, 2003; Westenbroek et al., 2004). The results regarding cell survival a more variable, with no effect (Pham et al., 2003; Heine et al., 2004) or decreased cell survival (Czeh et al., 2002; Westenbroek et al., 2004; Thomas et al., 2006) reported in males following chronic stress exposure. Similarly, in females chronic stress exposure results in decreased (Kuipers et al., 2006) or contradictory in an increase (Westenbroek et al., 2004) in cell survival (see *Table 2*). These findings demonstrate the necessity for more studies, particularly assessing the effect of chronic stress exposure on cell survival in males and females in parallel.

The observed sex differences in neurogenesis processes outlined above may at least partly be due to the fact that males and females differ in their sensitivity, and coping mechanisms, for stress (Dalla et al., 2008), as well as in basal (Galea et al., 1997) and stress-induced CORT levels (Shors, 2006b; Barha et al., 2007). In contrast to males, who habituate to chronic stress situations, females show a longer, more robust rise in CORT levels (Galea et al., 1997; Falconer and Galea, 2003) and express higher peak CORT levels in response to stress (McCormick and Mathews, 2007). This seems to be of particular importance, given the fact that increased glucocorticoid levels are believed to be one of the primary mediators underlying the detrimental effects of stress on adult hippocampal neurogenesis (for reviews see Mirescu and Gould, 2006; Joels et al., 2007). Persistent elevations of CORT by either exogenous administration or chronic stress exposure decrease cell proliferation, cell survival and differentiation of newborn cells in males (Gould et al., 1991b; Cameron and Gould, 1994; Wong and Herbert, 2006; Murray et al., 2008). In contrast, lowering CORT levels by

adrenalectomy (ADX) (Gould et al., 1991b; Cameron and Gould, 1994) or inhibiting the effect of CORT *via* blocking its receptors (Mayer et al., 2006; Oomen et al., 2007), is able to prevent the stress-induced decrease in neurogenesis. Although, the exact mechanisms underlying the negative effect of glucocorticoids on adult hippocampal neurogenesis are not fully identified, two possible mechanisms are commonly described in the literature. The first possibility is a direct effect by the binding of CORT to glucocorticoid and mineralocorticoid receptors (GRs and MRs, respectively) in the hippocampus. This theory is supported by the fact that chronic application of the GR agonist dexamethasone inhibits neural progenitor cell proliferation (Kim et al., 2004), whereas blockade of the MR receptor by spironolactone restores the detrimental stress effect on neurogenesis (Wong and Herbert, 2005). However, only a small percentage of progenitor cells (10-20%) in the DG express GRs and MRs (Cameron et al., 1993a; Garcia et al., 2004), which suggests that there exists an alternative pathway involved in the stress-mediated decrease in neurogenesis. Thus, the second postulated mechanism centres on glutamate, an excitatory neurotransmitter. Glutamate release within the hippocampus is increased *via* glutaminergic fibres that originate from the entorhinal cortex that converge on the DG *via* the perforant path (Abraham et al., 1998). Such increased excitatory neurotransmission decreases cell proliferation, whereas lesions in the pathway have the opposite effect (Gould et al., 1994; Cameron et al., 1995). Despite the array of studies assessing the effect of chronic stress on adult hippocampal neurogenesis in males and virgin females, there are only few studies that assess the effect of chronic stress on neuronal and astroglial differentiation patterns and stem cell quiescence. A detailed knowledge of these processes would lead to a better understanding of the consequences of chronic stress exposure. Further, to date no studies have assessed the effect of stress on neurogenic processes in postpartum females. Therefore, given the known influence of

glucocorticoids on the regulation of hippocampal neurogenesis, and the fact that the lactation period represents a time of attenuated stress responsivity, the lack of studies focussing on lactating females is astonishing. Another reason to presume that stress may affect neurogenesis in the peripartum period is the high prevalence of mood and anxiety disorders at this time (Robertson et al., 2004; Beck, 2006; Lonstein, 2007), coupled with the correlation between depression and neurogenesis (see Section 3.2) (for reviews see Pittenger and Duman, 2008; Hanson et al., 2011).

Table 2: Overview of how chronic stress affects hippocampal neurogenesis in male and female rats

Effect of chronic stress on:	**Males**	**Females**
Cell proliferation	↓ (Pham et al., 2003; Heine et al., 2004)	no effect (Tanapat et al., 2001; Falconer and Galea, 2003; Westenbroek et al., 2004)
Cell survival	no effect (Pham et al., 2003; Heine et al., 2004)	↑ (Westenbroek et al., 2004)
	↓ (Czeh et al., 2002; Westenbroek et al., 2004; Thomas et al., 2006)	↓ (Kuipers et al., 2006)

5. Consequence of early life stress on physiological and behavioural outcomes in adulthood

While the sections above have discussed the negative consequences of stress in adulthood, focusing especially on the peripartum period, it is also well-documented that adverse environmental events that occur during the prenatal and postnatal period represent a major risk factor for the development of psychopathologies later in life. Early life stress exposure has been linked with increased risk of adult psychopathologies, including anxiety and depression disorders, substance abuse and schizophrenia (Huttunen et al., 1994; Barnow et al., 2001; Heim and Nemeroff, 2001; Kalinichev et al., 2002; Meaney et al., 2002; Romeo et al., 2003; Howes et al., 2004; Fumagalli et al., 2007; Heim et al., 2008). Thus, there are numerous animal and human studies assessing the effect of early life stress on the offspring outcome in adulthood. A number of different gestational stress paradigms have been described to cause such detrimental effects on the offspring development, including repeated daily immobilization (Rojo et al., 1985; Maccari et al., 1995; Vallee et al., 1996), alteration of different variable stressors (Koenig et al., 2005; Emack et al., 2008) and overcrowding (Harvey and Chevins, 1985) (for reviews see Weinstock, 2001, 2005). Postnatal stress is generally induced by interference with mother-pup interactions. The early relationship between mother and child, including maternal care, is one important factor influencing the physiological and behavioural outcome of the offspring throughout all mammalian species; even under conditions of stress (Suchecki et al., 1993; Liu et al., 1997; Caldji et al., 1998; Meaney, 2001; Kaplan et al., 2008). Therefore, maternal separation (MS), which includes the removal of pups from the dam for a distinct period of time and days, is one of the most commonly used models of postnatal stress. Multiple variations of MS

procedures have been employed with variations in both the length (e.g. from 15 min up to 24 h) and the number of days (e.g. from 1 day up to 14 days) (Plotsky and Meaney, 1993; Biagini et al., 1998; Pryce et al., 2001; Rosztoczy et al., 2003; Arborelius and Eklund, 2007). While the mechanisms and timing of these stress paradigms differ, the behavioural and physiological consequences for the offspring show considerable similarities (for review see Pryce et al., 2001).

Animal studies assessing the effects of prenatal and postnatal stress revealed that chronic stress during that susceptible period does not only lead to alterations in basal physiological parameters like birth weight (Rice et al., 2006; Buhl et al., 2007; Rice et al., 2007; Emack et al., 2008), body weight gain (Buhl et al., 2007; Emack et al., 2008; Hillerer et al., 2011b) and adrenal morphology (Ward et al., 2000), but moreover changes in HPA axis activity. Both types of early life stress induce an increased HPA axis response to stress (Maccari et al., 2003; Seckl, 2004; Owen et al., 2005), which is at least partly due to a diminished negative glucocorticoid feedback of the hippocampus (Maccari et al., 1995; Vallee et al., 1996; Vallee et al., 1997). However, whereas both prenatally and postnatally stressed offspring show an altered CRH mRNA expression pattern in the PVN and the amygdala (Plotsky and Meaney, 1993; Cratty et al., 1995; Bosch et al., 2006; Bosch et al., 2007b), basal ACTH and CORT are affected by MS (Plotsky and Meaney, 1993), but not by prenatal stress (Maccari et al., 1995; Vallee et al., 1996; Vallee et al., 1997). Such abnormalities in HPA axis function are of particular importance with respect to the known association between HPA axis dysregulation and psychiatric disorders, including anxiety and mood disorders (Arborelius et al., 1999; de Kloet et al., 2006).

Chronic stress occurring either *in utero* or during the postnatal period induces a complex array of different behavioural abnormalities, suggesting that early life stress affects multiple

systems and circuitries involved in emotional, cognitive and decision-making processes and as such is not associated with a single mental disease (Fumagalli et al., 2007). Thus, early life stress (both pre- and postnatal) leads to anxiety-related and depression-like phenotypes in numerous different species, including rodents and humans (Vallee et al., 1997; Alonso et al., 1999; Ward et al., 2000; MacQueen et al., 2003; Morley-Fletcher et al., 2003; O'Connor et al., 2003; Rice et al., 2007; Darnaudery and Maccari, 2008). Moreover a change in the expression of social behaviour is a common consequence of early life stress throughout all species studied to date; although these findings are not as consistent and well studied as the aforementioned behavioural parameters. However, there are studies revealing an increase in intermale aggression and juvenile play fighting in rats (Veenema et al., 2006; Veenema and Neumann, 2009), as well as a reduction in locomotion and contact seeking and an increase in stereotypic behaviours (i.e. pacing, digit sucking, cage shaking and bouncing) in nonhuman primates (Dettling et al., 2002; Feng et al., 2011) after exposure to adverse early life events. Similar deficits in social behaviour are observed in early neglected children, who show abnormal identification and response to facial expressions (Pollak et al., 2000), as well as increased aggression (Chen et al., 2011).

The observed changes might be, at least partly, due to the fact that brain regions like the amygdala, the prefrontal cortex and the hippocampus, all known for their importance in mediating behavioural processes, undergo profound prenatal and postnatal neuronal developmental processes, including synaptogenesis, neurogenesis and dendritic development (for reviews see Weinstock, 2005, 2008). Thus, both prenatal and postnatal stress are associated with a decrease in hippocampal neurogenesis in rats and monkeys (Lemaire et al., 2000; Coe et al., 2003; Mirescu et al., 2004; Lucassen et al., 2009; Hulshof et

al., 2011). As stated above, such alterations in offspring behaviour can be used to verify the successful employment of novel peripartum stress models.

Although the findings outlined above suggest that the effects of pre- or postnatal stress are largely in accordance, the majority of such studies have only examined stress exposure during one of the time points. Therefore, it would be of major interest to assess the behavioural outcome, particularly social aspects, within studies that assess pre- and postnatal stress paradigms simultaneously. Additionally, the vast majority of studies do not evaluate the effect of the stress procedure on maternal adaptations, such as maternal care, which could significantly modify the consequences of gestational stress or separation stress on the offspring.

6. Aim of the present thesis

Given the outlined knowledge in the sections above, the aim of my thesis was:

1) To establish an animal model of chronic peripartum stress

2) To assess the effects of chronic stress during the peripartum period on common adaptations

3) To investigate if the stress-induced changes are specific to the peripartum period

4) To determine whether the stress-induced alterations in peripartum adaptations affect the physiological and behavioural outcome of the offspring

7. Outline of the present thesis

Chapter 2 describes the effect of chronic stress during pregnancy on known peripartum-associated neuroendocrine and molecular adaptations and their relation to the behavioural outcome of the dam.

Chapter 3 investigates basal and chronic stress-induced sex differences in adult hippocampal neurogenesis, including cell proliferation and survival, neuronal and astroglial differentiation patterns, as well as stem cell quiescence. These findings are related to the consequences of stress exposure on CORT levels.

Chapter 4 describes peripartum-associated changes in adult hippocampal neurogenesis. Furthermore it assesses the effect of chronic stress during early lactation on changes in cell proliferation, cell survival, differentiation and stem cell quiescence.

Chapter 5 directly compares the effect of two different types of early life stress, prenatal and postnatal stress on anxiety, depression-like and social behaviour in adulthood. Moreover, it determines if the amount of maternal care affects the behavioural outcome seen in adulthood.

Chapter 2

Exposure to chronic pregnancy stress reverses peripartum-associated adaptations: implications for postpartum mood and anxiety disorders

Author's contribution:

Hillerer: Study design, performing of experiments, analyzing data, writing the manuscript

Reber: Establishment of adrenal analysis, performing experiments

Neumann: Study design, contribution to manuscript writing

Slattery: Study design, performing experiments, contribution to manuscript writing

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ABSTRACT

Maternal adaptations, such as decreased anxiety and attenuated stress-responsiveness, are necessary to enable successful postnatal development of the offspring. However, there is growing evidence that they are also required to protect the mental health of the mother, and that exposure to chronic stress during pregnancy may prevent such adaptations. Overcrowding stress (OC) (24 h) and restraint stress (RS) (2 x 1 h) were employed on alternate days between PD4 - PD16 to examine the impact of chronic pregnancy stress on relevant behavioural, neuroendocrine and neuronal peripartum adaptations. To determine whether the chronic stress-induced alterations were specific to the peripartum period we included virgins as controls. Validating the stress procedure, we demonstrated decreased body-weight gain and increased adrenal weight in stressed dams, relative to their unstressed controls. Chronic stress prevented a number of peripartum adaptations, including basal plasma hyper-CORT levels, increased OXT mRNA expression in the hypothalamic PVN and anxiolysis. However, chronic stress did not prevent the peripartum-associated decrease in CRH mRNA expression, attenuated CORT response to an acute stressor nor did it affect hypothalamic AVP mRNA expression. Illustrating the specificity of these stress-induced changes to the peripartum period, none of these parameters were affected in stressed virgins. While chronic stress did not alter depression-like behaviour, it reversed the response to acute imipramine-treatment and increased active maternal behaviour in lactation. Thus, prevention of the peripartum-associated increases in basal CORT and OXT system activity by pregnancy stress reveal two alterations that may increase the risk of postpartum psychiatric disorders, specifically anxiety.

INTRODUCTION

Across all mammalian species the peripartum period is characterised by behavioural, neuroendocrine and neuronal adaptations, which prepare a mother for the impending birth and nurturance of the offspring (Brunton et al., 2008; Slattery and Neumann, 2008). While these adaptations are essential to ensure the survival and health of the offspring (Carter et al., 2001), we hypothesize that they are also required for maternal mental health (Neumann, 2003; Slattery and Neumann, 2008). Thus, although anxiolysis, enhanced calmness and attenuated stress-responses of the HPA axis are observed in the majority of mothers, a significant percentage display increased vulnerability to mood and anxiety disorders, such as postpartum anxiety (5-12%; Lonstein, 2007), postpartum depression (PPD, 5-25%; Beck, 2006), or the rarer postpartum psychosis (0.1%; Bridges, 2008; Jones et al., 2008). A number of risk factors are known for postpartum mood and anxiety disorders including smoking (Beck, 2006; Friedman and Resnick, 2009) and alcohol abuse (Homish et al., 2004), but also a history of anxiety and depression episodes (Heron et al., 2004; Neumann et al., 2005a), the social status of the mother (Hall and Holden, 2008; Soderquist et al., 2009), and stressful events during pregnancy (Robertson et al., 2004). Despite the high incidence of these disorders, and the detrimental outcome for both mother and child, their aetiology remains poorly understood, due in part to the lack of appropriate animal models.

The physiologically-occurring peripartum adaptations, which have been identified in pregnant and lactating animals and women in recent years, include basal hypercorticism / hypercortisolism (Lightman et al., 2001) as well as elevated levels of corticosterone binding globulin (CBG), which reduces the availability of free CORT (Koch, 1969; Pearlman, 1983; Douglas et al., 2003). However, a concurrent attenuation of the responsiveness of the HPA

axis to acute stressors during the peripartum period has also been shown in rodent and human studies (Stern et al., 1973; Neumann et al., 1998b; Neumann et al., 2000a; Lightman et al., 2001; Kammerer et al., 2002). The reduced stress responsivity is mediated *via* numerous brain mechanisms including alterations in the inhibitory and excitatory pathways that act within the hypothalamus. For example, the activity of inhibitory systems, such as OXT and PRL (Douglas and Russell, 1994; Douglas et al., 1998; Neumann et al., 2000b; Altemus, 2006); (for review see Neumann, 2008; Slattery and Neumann, 2008) is increased, while excitatory pathways including the noradrenergic system are decreased in the peripartum period. Thus, hypothalamic expression and release of OXT and PRL are increased, while the basal noradrenergic tone within the PVN is decreased, consequently resulting in decreased CRH expression (Toufexis et al., 1998; Douglas et al., 2005; Slattery and Neumann, 2008). Additionally, during lactation increased expression of AVP within the PVN has been observed, which has also been hypothesized to mediate, at least in part, the increased basal CORT levels (Walker et al., 2001b). These neurochemical changes may also underlie the behavioural alterations observed in the peripartum period, which include reduced anxiety (Carter et al., 2001; Bosch et al., 2005; Slattery and Neumann, 2008), calmness (Heinrichs et al., 2001), as well as increased maternal care (Rosenblatt, 1994) and maternal aggression (Erskine et al., 1978; Lonstein and Gammie, 2002; Bosch et al., 2006). Thus, it is feasible that alteration, or even prevention of these normal adaptations may underlie postpartum mood and anxiety disorders, and exposure to chronic stress in pregnancy is a known risk factor for these disorders. In contrast to the effects on the offspring (Weinstock, 2008), basic research on the consequences of chronic stress in pregnancy on the mother is largely lacking (Neumann et al., 2005a). This is due to a lack of appropriate animal models for studying

postpartum mood and anxiety disorders (Slattery and Neumann, 2008), but also reflects the general lack of research assessing the effect of chronic stress in females.

Therefore, we aimed to characterise the effects of a novel chronic psycho-social stress paradigm during pregnancy on established peripartum adaptations. As such, we hypothesized that exposure to chronic stress in pregnancy would, at least partly, prevent behavioural, neuroendocrine and neuronal adaptations specific to the reproductive status of the female. Therefore, we included virgin groups as controls for both stress- and peripartum-induced alterations.

MATERIALS AND METHODS

Animals

Female Wistar rats (Charles River, Sulzfeld, Germany; 200-250g) were housed in groups of four in standard polycarbonate rat cages and allowed to habituate for at least seven days. All rats were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00h, 22°C ± 1°C, 60 ± 5% humidity) and had free access to water and standard rat diet. All experimental procedures were performed between 08:00 – 12:00h (except maternal observations - see below), all were approved by the Committee on Animal Health and Care of the local government of the Oberpfalz, and complied with international guidelines on ethical use of animals.

Mating procedure and confirmation of pregnancy

After habituation, all female rats were mated (two to three females/male); and pregnancy was verified by vaginal smears (designated PD0). To rule out any possible effect of mating on subsequent readouts, non-pregnant rats were assumed to be nulliparous (equates to "virgin" in the following text). All animals were returned to group cages with 2 virgin and 2 pregnant females housed together (cage size 55 x 35 x 20 cm) until PD4, the onset of the chronic stress procedure. Unstressed controls remained in these groups of 4 until PD16 (or equivalent in virgins) when they were single-housed. On the day of birth, i.e. lactation day (LD) 1, the number of pups in the litter and the average birth weight were determined, and then all litters were culled to 8 pups to ensure comparable conditions across all dams.

Stress procedure

From PD4 to PD16, stressed rats were exposed alternatively to daily RS (2 x 1 hr at least 3 h apart during light phase; plexiglass column with ventilation holes; 12 cm diameter) and OC (4 unfamiliar rats for 24 h; cage size: 40 x 25 x 15 cm) (see also *Figure 2*). RS and OC stress are both effective stressors in female rats when administered alone (Brown and Grunberg, 1995; Baranyi et al., 2005; Neumann et al., 2005a). Rats were single-housed on RS days and taken from individual housing into OC conditions the following day, resulting in social instability, which is a potent stressor in females (Herzog et al., 2009). Social stressors elicit a greater CORT response than RS (Koolhaas et al., 2011) and, thus, the RS in our paradigm was employed to complement the effect of social instability. From PD16 (or equivalent in virgins) the stressed rats were also kept in single-house conditions.

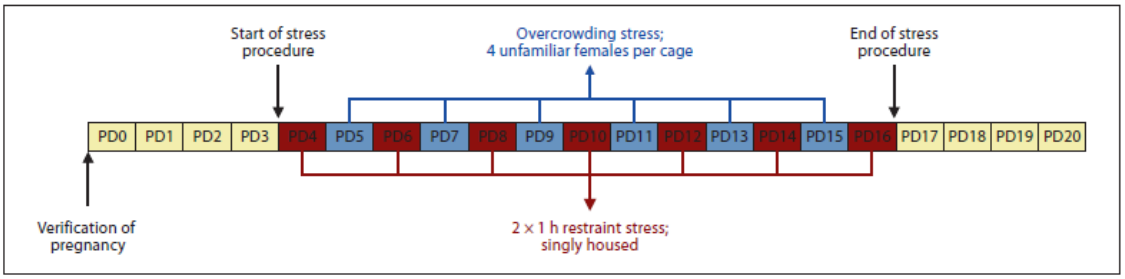

Figure 2: Schematic representation of the chronic pregnancy stress paradigm

The chronic psycho-social stress paradigm used between PD4 and PD16 for our own peripartum studies. Female rats (pregnant or virgin) were housed in cages of 4-5 until PD4 when the stress procedure started. On even days between PD4 and PD16 (in red), rats were weighed, housed individually and subjected to 2 x 1 h RS in a plexiglass tube (12 cm in diameter, with ventilation holes). On odd days (in blue), rats were weighed and transferred to overcrowded conditions, consisting of 4 pregnancy stressed rats in a small cage (40 x 25 x 15 cm). From PD17 onwards animals were singly housed. Control pregnant and virgin rats maintained in cages of 5 throughout the experiment until transferred to single housing on PD17 and weighed daily to mimic the handling conditions of the stressed group.

Validation of the stress paradigm

In the initial study, chronic stress-relevant parameters of the dams and the offspring were assessed to validate the novel stress paradigm. The stress paradigm was performed as above. Between LD1 and LD8, the pups were weighed daily after observation of maternal behaviour was finished (see below). On LD8, dams were weighed, killed by decapitation following CO_2 anaesthetization and adrenal glands and the thymus were taken out, pruned of fat tissue, and weighed by an experimenter blind to group, and relative adrenal weight (total weight of left plus right adrenals / body weight in mg/g), absolute thymus weight and body weight were analysed.

Ultrasonic vocalisations (USV) of prenatally stressed and non-stressed pups were determined by a bat detector (22kHz; BVL, Klein Goernow) on LD8 as previously reported (Winslow and

Insel, 1991; Landgraf and Wigger, 2002; Bosch et al., 2006; Brudzynski, 2009). Briefly, pups were taken out of the dams' homecage, brought to another room and placed on a round petri-dish (12 cm in diameter). Their USVs were scored for 10 min by an experienced observer blind to group and used as a measurement of anxiety in the pups (Landgraf et al., 2007).

HPA axis (re)activity

In order to determine chronic stress-induced changes in basal and acute stress-induced ACTH and CORT levels, a jugular-vein catheter was implanted on LD2 (or equivalent in virgins) under isoflurane anaesthesia as previously described (Neumann et al., 1998b). Following surgery, rats were single-housed until the end of the experiment. On LD8, i.e. 6 days after surgery, basal concentrations of ACTH and CORT as well as the neuroendocrine response to an acute stressor (forced swimming; 60 s, 30 cm water, 25 ± 1°C) were assessed. In detail, two basal blood samples (basal sample 1: 0.6ml; basal sample 2: 0.2ml), 30 min apart, substituted with sterile 0.9% saline, were taken 2 h after lights on and, at least, 90 min after attaching the catheter to tubing filled with heparinised saline (30 IU/ml; 0.2ml for the detection of ACTH and CORT levels). Thirty min after taking the second basal sample the rats were exposed to the swim stress. Further blood samples were taken 5, 15, 30 and 60 min after swim stress.

Plasma ACTH and CORT were quantified by radioimmunoassays using commercially available kits (DRG Instruments GmbH; Marburg) according to the manufacturer's protocol (Neumann et al., 2005a). Due to analytical errors, samples from one virgin stressed and one lactation

non-stressed rat were removed from the ACTH analyses, while the samples of one lactation stressed animal were removed from the CORT analyses.

Hypothalamic AVP, CRH and OXT mRNA expression

In a separate cohort, we examined the effects of pregnancy stress on basal AVP, CRH and OXT mRNA expression within the hypothalamic PVN at the end of pregnancy - a time of altered gene expression (Slattery and Neumann, 2008). Therefore, the brains of stressed and control PD20 and virgin rats were rapidly removed under short (10 sec) isoflurane anaesthesia, and flash-frozen 16-µm cryocut sections were slide mounted. The PVN was targeted using histological staining in combination with a brain atlas (Paxinos et al., 1985). Once the most rostral region of the PVN was visible, six slides containing six slide-mounted brain sections per animal were processed. One set each was used for the AVP, CRH and OXT mRNA *in situ* hybridizations, which were performed as previously described (Bosch et al., 2005; Bosch et al., 2007b). Briefly, for detection of AVP, CRH and OXT mRNA, highly specific, 48-single base, ^{35}S-labeled oligonucleotide probes were used (AVP: 5' GCA GAA GGC CCC GGC CGG CCC GTC CAG CTG CGT GGC GTT GCT CCG GTC 3'; CRH: 5' GGC CCG CGG CGC TCC AGA GAC GGA TCC CCT GCT CAG CAG GGC CCT GCA 3'; OXT: 5' CTC GGA GAA GGG AGA CTC AGG GTC GCA GGC GGG GTC GGT GCG GCA GCC 3'). Following *in situ* hybridization, the air-dried sections were exposed to BioMax MR films (Eastman Kodak, Rochester, New York, USA) for 2 h (OXT) or 7 days (AVP and CRH). AVP, CRH and OXT mRNA expression were measured as grey density with a computerized image program (ImageJ 1.31, National Institutes of Health, http://rsb.info.nih.gov/ij/). Brain slices which contained comparable sections of PVN were measured for each subject to provide individual means, and only those

with at least 4 (out of 6) measurable sections were included in statistical analyses. We did not distinguish between the magno- and parvo-cellular regions of the PVN. Background activity was automatically subtracted from measured areas to yield values for specific binding.

Maternal behaviour

In a separate cohort, maternal behaviour was assessed in stressed and non-stressed dams daily between LD2 and LD7, except on LD3. According to a time-sampling protocol (Bosch and Neumann, 2008), undisturbed maternal behaviour was observed for 5-10 s every second minute in the light phase between 06:00h and 10:00h continuously, and from 11:00h-12:00h, 13:00h-14:00h and 17:00h-18:00h, as well as one hour in the dark phase (18:00h-19:00h). The pup-directed parameters assessed were arched back nursing (ABN; quiescent kyphotic nursing), blanket behaviour, laying on side or back. Moreover, we assessed licking and grooming pups; pup carrying, digging, locomotion, rearing, eating/drinking, self-grooming and sleeping. The frequency of the recorded behaviour, e.g. ABN, was calculated as the mean total ABN per day.

Anxiety-related behaviour

The EPM was performed as previously described (Slattery and Neumann, 2010) on LD8 in a separate cohort of animals. Briefly, the 5-min test was performed on a plus-shaped maze, which was elevated (70 cm) from the floor and consisted of two closed arms (50 x 10 x 40 cm; 25-30 lux) and two open arms (50 x 10 cm; 90-100 lux) separated by a central neutral zone (10 x 10 cm). A camera above the maze enabled assessment of behaviour. The test

started by placing the animal in the neutral zone facing a closed arm and the percentage time spent on the open arms, as an indicator of anxiety, was determined by an observer blind to treatment. The number of closed arm entries was used as an indicator of locomotor activity.

Depression-like behaviour

A separate cohort of only lactating stressed and unstressed animals was used in order to assess the effects of chronic pregnancy stress and acute imipramine administration on depression-like behaviour in lactation (Slattery et al., 2005), as a preliminary study indicated that the chronic stress paradigm did not alter FST behaviour in virgin or lactating rats (data not shown). Imipramine has been repeatedly shown to increase climbing behaviour in the FST, which is decreased in lactation due to the reduced noradrenergic drive at this time-point. Thus, the experiment aimed to determine whether stress altered the response to its administration. Briefly, rats were individually placed into a plexiglass cylinder filled with water (30 x 49 cm; 25 ± 1°C; 30 cm deep) on LD8 for 15 min, towel-dried, and returned to their home cage. Water was changed between each test. Twenty-four hours later, the rats were replaced in the swim cylinder for 5 min. Animals were injected with vehicle (0.9 % saline; i.p 1ml/kg) or imipramine (20 mg/kg; (Slattery et al., 2005) 23.5, 5 and 1 h before the 2nd swim session. Both sessions were video-recorded for subsequent analysis. Using a time sampling technique, the predominant behaviours, climbing, swimming or immobility, were assessed by an observer blind to treatment in each 5-s period of the test exposure. Climbing behaviour consisted of upward-directed movement of the forepaws usually along the side of the swim cylinder, swimming behaviour consisted of horizontal movement, and immobility

was defined as floating in the water only making movements necessary to maintain its head above the water (for pictorial representations of the behaviours see Cryan et al., 2002).

Statistical analysis

Results were analysed using either an unpaired Student's t-test, a Mann Whitney *U* test, or a one- or two- way analysis of variance (ANOVA) with or without repeated measures, as appropriate. Any statistical differences, which were set at $p \leq 0.05$, were further analysed using a Fisher's *post-hoc* test. Data are expressed as mean ± S.E.M. Statistical analyses were performed using SPSS for Windows (version 16; SPSS Inc, Chicago, IL, USA).

RESULTS

Chronic stress effects on physiological parameters in dams

To confirm the validity of the novel chronic stress procedure a number of stress-related physiological parameters of the females were examined. Chronic stress was shown to reduce body-weight gain (factor stress; $F_{1,43}=70.8$; $p<0.001$) with *post-hoc* analyses confirming a reduced weight gain in both stressed virgin and peripartum groups compared with their respective non-stressed controls ($p<0.001$; Fig. 3A). Additionally, stressed animals showed an increased relative adrenal weight (factor stress; $F_{1,43}=6.31$; $p=0.016$), which was significant

between stressed and non-stressed dams in the *post-hoc* analyses ($p<0.05$; Fig. 3B). Chronic stress did not alter thymus weight (data not shown).

Chronic pregnancy stress effects on offspring parameters

Pregnancy stress had no effect on pup litter size (non-stressed (NS): 12.4 ± 1.1, n=10; stressed (S): 13.6 ± 0.4, n=9; t_{17} = -0.732; p=0.47) or birth weight (NS: 6.6 ± 0.2g, n=10; S: 6.1 ± 0.1g, n=9; t_{17} = 1.918; p=0.07). However, there was a significant difference in postnatal development of body weight between stressed *versus* non-stressed offspring from LD2 onward ($F_{1,17}$=4.45; p=0.05; Fig. 3C) with a lower body weight observed in prenatally stressed offspring on LD2, 6, 7 and 8 ($p<0.05$). Furthermore, pups of stressed mothers showed an increase in the number of ultrasonic calls per 10 min compared with those of non-stressed mothers (NS: 345 ± 37, n=5; S: 762 ± 42, n=6; t_9 = -6.86; $p<0.001$; data not shown).

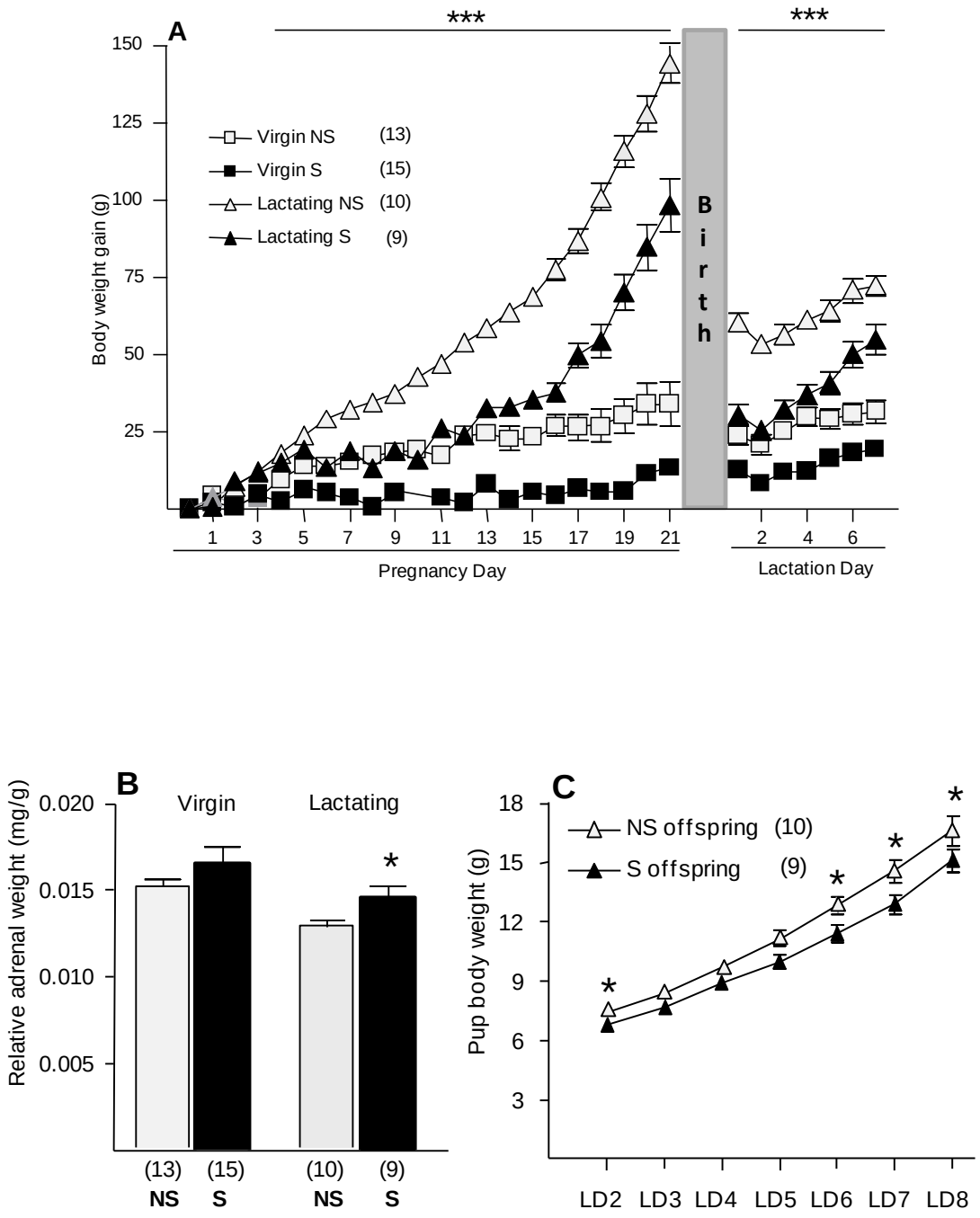

Figure 3: Chronic stress effects on physiological parameters

Exposure to chronic psycho-social stress (PD4 – PD16 or equivalent in virgin animals) led to a decreased body weight gain in virgin and peripartum rats (A) and an increased relative adrenal weight LD8 in the stressed-peripartum group (B). Offspring of stressed dams also displayed a decreased body weight on postnatal days 2, 6, 7 and 8 (C). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. * $p < 0.05$; ***, $p < 0.001$ *vs.* respective non-stressed control groups. LD; Lactation Day, NS; non-stressed, S; stressed

Chronic stress effects on plasma ACTH and CORT levels

While a significant rise in ACTH secretion was observed following 60s acute swim stress (factor time x acute stress; $F_{4,172}$=59.2; p<0.001), there was no effect of reproductive status ($F_{1,43}$=0.01; p=0.9) or pregnancy stress ($F_{1,43}$=0.26; p=0.6) on this response (Fig. 4A). Separate analyses revealed that basal ACTH levels (expressed as the mean of two basal samples taken 30 min apart) did not differ with respect to status ($F_{1,43}$=0.85; p=0.36) or chronic stress ($F_{1,43}$=1.1; p=0.31; data not shown) nor did the area under the curve differ (factor status; $F_{1,43}$=0.02; p=0.9; factor chronic stress; $F_{1,43}$=0.3; p=0.6; data not shown).

Repeated-measures ANOVA revealed that the CORT response to swim was attenuated in lactation compared with virgins (factor time x status; $F_{4,176}$=7.84; p<0.001; Fig. 4B), but there was no effect of chronic stress on this response ($F_{1,44}$=0.5; p=0.8; Fig. 4B). Separate analyses of basal CORT levels (expressed as the mean of two basal samples taken 30 min apart) revealed a significant interaction between reproductive status and chronic stress ($F_{1,44}$=6.23; p=0.016). *Post-hoc* analyses revealed a basal hyper-CORT level in unstressed lactating dams (p<0.001), which was prevented by pregnancy stress (p<0.05; Fig. 4C). Assessment of the area under the curve revealed an attenuated response in lactating rats compared with virgins ($F_{1,44}$=8.48; p=0.006) but no chronic stress ($F_{1,44}$=0.89; p=0.8) or interaction effects ($F_{1,44}$=0.22; p=0.6; Fig. 4D).

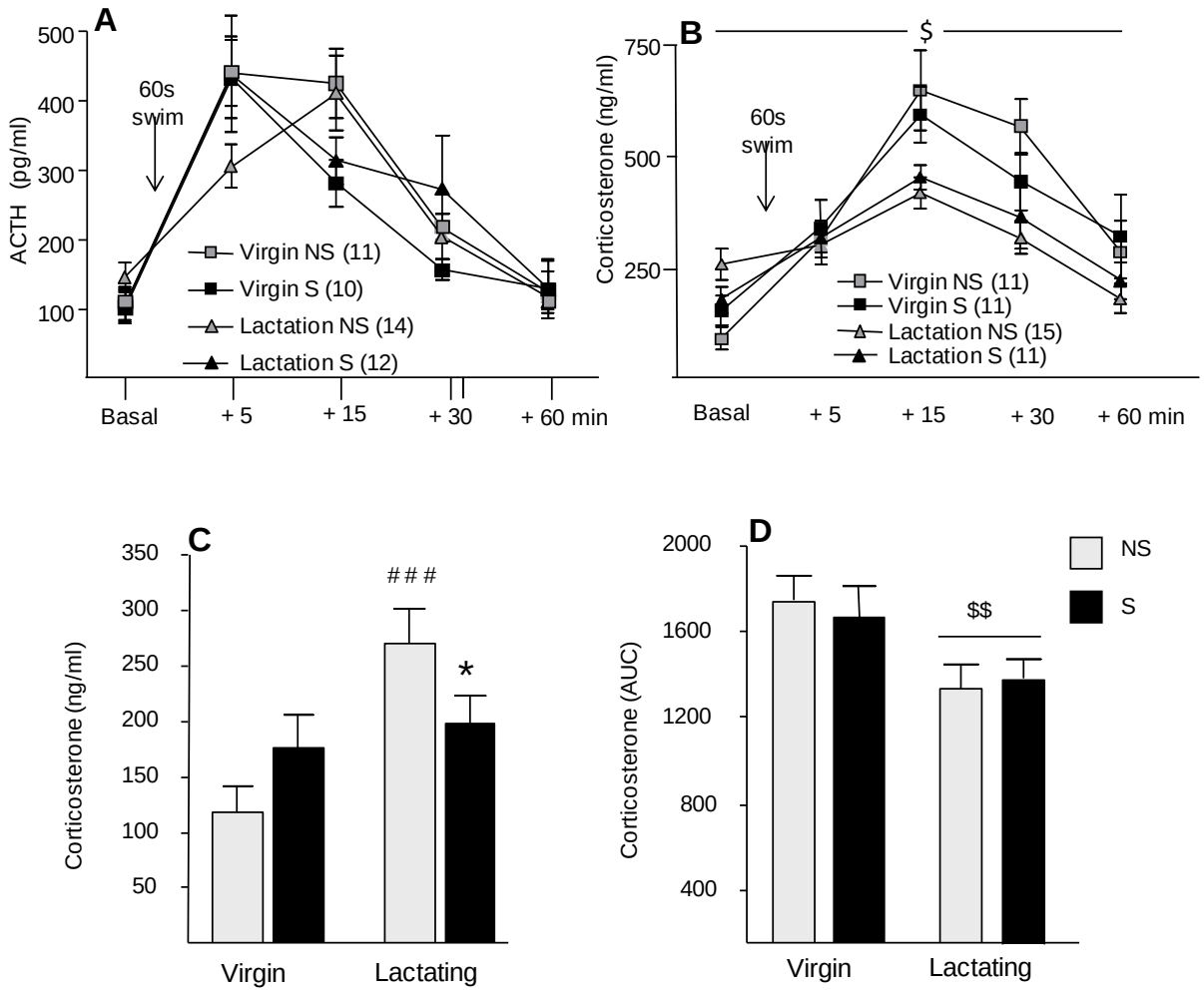

Figure 4: Chronic stress effect on plasma ACTH and CORT levels

Effect of chronic psycho-social stress (PD4 – PD16 or equivalent in virgin animals) on basal (expressed as the mean of two basal samples taken 30 min apart) and 60 s acute swim stress-induced plasma ACTH and CORT concentrations as assessed on LD8 (or equivalent in virgin animals). No effect of reproductive status or pregnancy stress was observed on basal or acute stress-induced plasma ACTH levels (A). Lactation was associated with an attenuated CORT response to a 60s swim stress compared with virgins (B), but no effect of chronic stress was observed (B). Basal CORT levels were elevated in lactating non-stressed dams *versus* virgins, which was prevented by pregnancy stress (C). Assessment of the CORT area under the curve (AUC) revealed that dams had an attenuated stress-induced rise compared with virgins (D) Data represent mean ± SEM with numbers in parentheses representing the group sizes (group sizes in C and D are identical to those shown in B). *, p< 0.05 *vs.* respective non-stressed group; ###, p< 0.001 *vs.* equivalent virgin group; $, p< 0.05 and $$, P< 0.01 *vs.* virgin rats (main ANOVA effect). AUC; area under the curve, NS; non-stressed, S; stressed.

Chronic stress effect on hypothalamic AVP, CRH and OXT mRNA expression

AVP mRNA expression within the PVN was not altered by pregnancy (factor status; $F_{1,37}$=0.87; p=0.4) or chronic stress ($F_{1,37}$=0.8; p=0.4; Fig 5A). While CRH mRNA expression within the PVN was found to be dependent on reproductive status ($F_{1,39}$=28.8; p<0.001; Fig. 5B), it was not altered by chronic stress ($F_{1,39}$=1.39; p=0.3). *Post-hoc* analyses revealed that CRH mRNA expression was lower in both lactation groups compared with their respective controls (p<0.01; Fig. 5B). OXT mRNA expression within the PVN was found to be dependent on reproductive status ($F_{1,43}$=5.68; p=0.02; Fig. 5C) but not chronic stress ($F_{1,43}$=0.89; p=0.3). *Post-hoc* analysis revealed a higher OXT mRNA expression in non-stressed PD20 rats compared with virgins (p<0.01; Fig. 5C) but no difference between the stressed groups. Separate analysis revealed that pregnancy stress prevented the rise in OXT mRNA levels seen in non-stressed PD20 rats (Mann Whitney *U*; p<0.05; Fig. 5C; with representative OXT autoradiograms shown in Figure 5D).

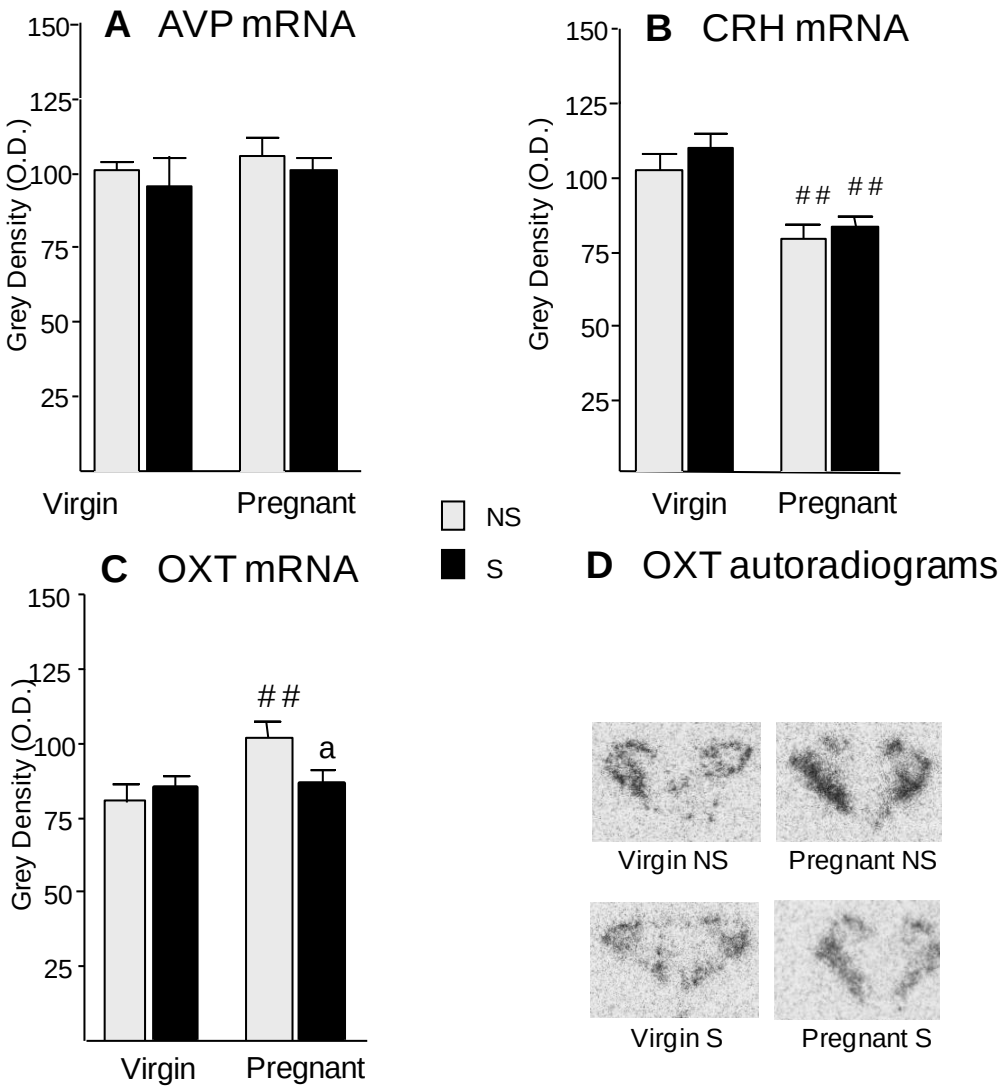

Figure 5: Chronic stress effects on hypothalamic AVP, CRH and OXT mRNA expression

Effect of chronic psycho-social stress (PD4 – PD16 or equivalent in virgin animals) on AVP mRNA expression (A), CRH mRNA expression (B) and OXT mRNA expression (C) within the hypothalamic PVN, when assessed on PD20 (or equivalent in virgin animals). Expression of AVP mRNA within the PVN was not altered by pregnancy or stress (A). However, CRH mRNA expression in the PVN was dependent on the reproductive status, with a decreased expression observed in both pregnant groups compared with their respective controls (B). No effect of chronic stress was observed on CRH mRNA expression. OXT mRNA expression within the PVN was increased at the end of pregnancy *versus* virgins (C), and separate analyses revealed that chronic pregnancy stress prevented this rise (C), which is shown in the representative images (D). Data represent mean ± SEM (n=9-12). ##, p< 0.01 *vs.* equivalent virgin group; a, p<0.05 *vs*. non-stressed pregnant group using a Mann Whitney U test. NS; non-stressed, S; stressed

Chronic pregnancy stress effect on maternal care

Chronic psycho-social stress in pregnancy resulted in a change in active maternal care as seen by an increase of ABN across the light-phase (factor stress; $F_{1,28}$=4.39; p=0.05; Fig. 6A). Repeated-measures ANOVA revealed a significant difference across the entire observation period. *Post-hoc* analyses revealed a trend towards more ABN in stressed dams compared with non-stressed dams on all days, which reached significance on LD2 (p<0.05; Fig. 6A). There was also a decrease in ABN across lactation (factor day; $F_{4,112}$=20.6; p<0.001) but no interaction effect was observed (factor day x stress; $F_{4,112}$=0.64; p=0.6). No difference was observed in ABN during the dark phase (data not shown).

Chronic stress effects on anxiety- related behaviour

A significant interaction between reproductive status and chronic stress on the percentage time spent on the open arms of the EPM was observed (factor status x stress; $F_{1,34}$=6.40; p=0.02; Fig. 6B). *Post-hoc* analyses revealed that chronic stress increased anxiety-related behaviour in lactating (p<0.01), but not in virgin animals (p>0.05). Separate analyses confirmed the lactation-associated anxiolysis (Mann Whitney *U*; p=0.04; Fig. 6B). The number of closed arm entries, indicative of locomotor activity, was not altered by the stress procedure ($F_{1,34}$=2.1; p=0.2; Fig. 6B).

Effects of chronic pregnancy stress on depression-like behaviour

A significant interaction between chronic stress exposure and imipramine treatment was observed on climbing ($F_{1,25}$=4.78; p=0.04) and immobility ($F_{1,25}$=5.74; p=0.02) behaviour, but

not on swimming ($F_{1,25}$=0.19; p=0.7; Fig. 6C) in the FST. *Post-hoc* analyses revealed no difference between stressed and non-stressed vehicle groups, but a trend towards increased climbing in the stressed group (Fig. 6C). Acute imipramine treatment increased climbing with a concurrent decrease in immobility in the non-stressed dams compared to equivalent vehicle-treated group ($p<0.05$; Fig. 6C). Stressed imipramine-treated animals climbed less ($p<0.05$) and were more immobile ($p<0.05$) than the non-stressed imipramine group.

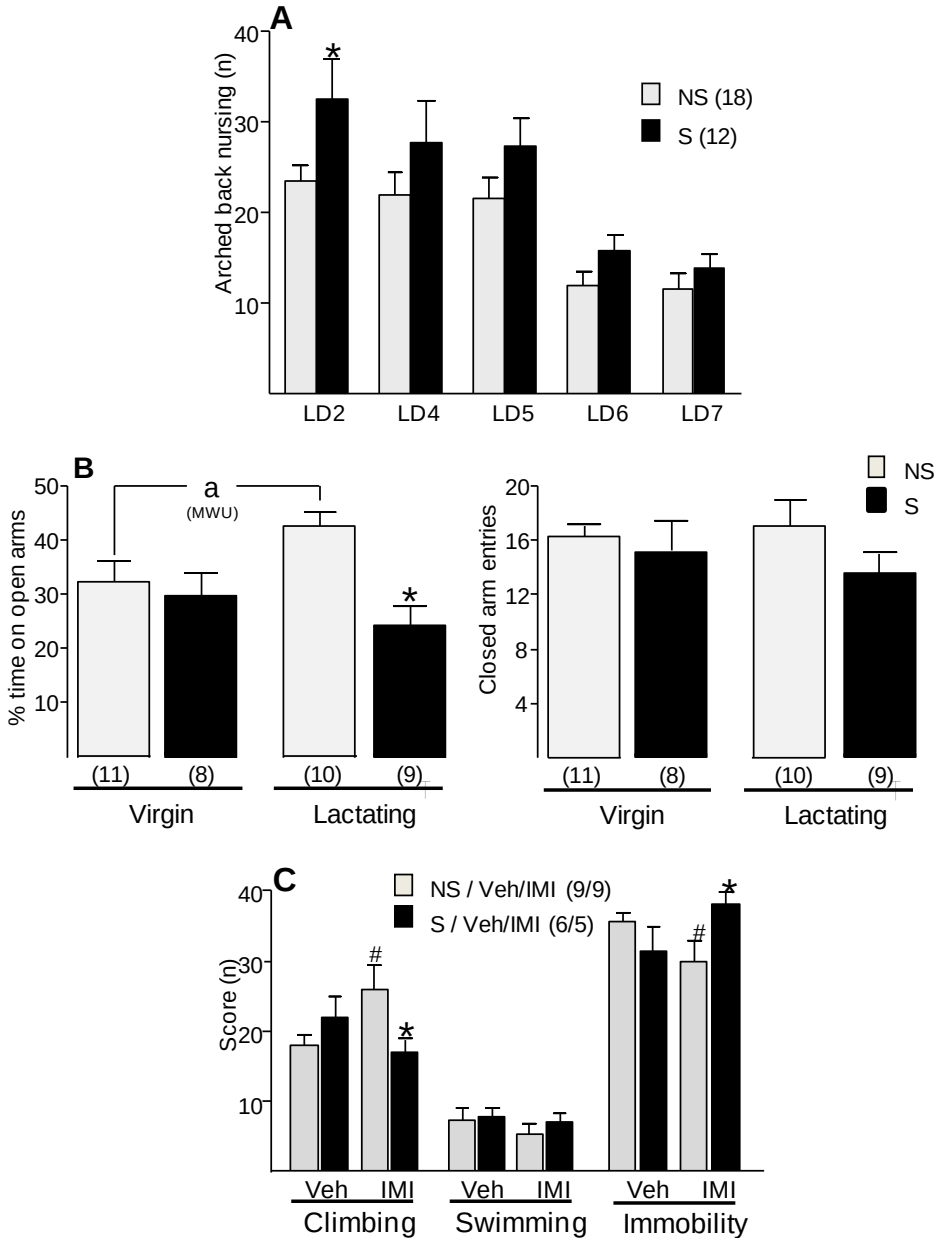

Figure 6: Chronic stress effects on maternal care, anxiety-related and depression-like behaviour

Exposure to chronic psycho-social stress (PD4 - PD16 or equivalent in virgin animals) increased ABN. Repeated-measures ANOVA revealed a significant difference in ABN between the groups across the entire time and subsequent *post-hoc* analyses showed a significant difference on LD2 between stressed and non-stressed dams (A). Moreover, exposure to chronic psycho-social stress reduced the percentage time spent on the open arms of the plus-maze on LD8 (B) without altering the number of closed arm entries on the EPM. Separate analyses confirmed the lactation-associated anxiolysis in non-stressed dams compared with non-stressed virgins (B). No effect of chronic pregnancy stress was observed on any behavioural parameter assessed in the FST (C). However, imipramine (20mg/kg; i.p.) given 23.5, 5 and 1 hr before the test increased climbing with a concurrent decrease in immobility in non-stressed dams. Pregnancy-stressed imipramine-treated animals climbed less and were more immobile (C) than the non-stressed imipramine group. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.05$ *vs.* respective non-stressed group; #, $p< 0.05$ *vs.* respective vehicle-treated group. a, $p<0.05$ *vs.* respective virgin group using a Mann Whitney U test. MWU; Mann Whitney U test, NS; non-stressed, S; stressed.

DISCUSSION

The present study was designed in order to gain a better insight into the effects of pregnancy stress on peripartum adaptations. After validation of the stress model, we demonstrated that chronic pregnancy stress exposure prevented the basal hyper-CORT levels observed during lactation, but did not significantly affect the attenuated HPA axis response to an acute stressor. At the neuronal level, chronic stress did not affect AVP or CRH mRNA expression, but prevented the peripartum-induced rise in OXT mRNA within the PVN. These physiological and molecular changes were accompanied by alterations in behaviour, with stress preventing lactation-associated anxiolysis and increasing the time spent actively nursing. These results suggest that pregnancy stress significantly interferes with numerous peripartum adaptations, which may lead to a greater susceptibility for the development of post-partum mood and anxiety disorders. In further support of their specificity, the stress-induced effects were only observed in the peripartum group.

Validation of the pregnancy stress model

The effects of chronic psycho-social stress on physiological parameters, such as decreased body weight gain, increased adrenal weight and decreased thymus weight have been well-documented in male mice (Reber et al., 2006; Reber and Neumann, 2008) and rats (Bielajew et al., 2002; Baranyi et al., 2005; Qi et al., 2006; Marin et al., 2007; Rygula et al., 2008; Herzog et al., 2009). In our stress model, the decreased body weight gain during pregnancy and lactation, together with the increase in the relative adrenal weight is consistent with

these findings in males. In contrast, while virgin animals showed a stress-related decrease in body weight, no difference in adrenal weight was observed. Given the fact that the adrenals were assessed more than 2 weeks after stress termination, this suggests that the alterations are longer-lasting in the peripartum period, or that dams are unable to recover from the stress exposure due to the demands of the offspring.

Moreover, with respect to pregnancy stress, changes in physiological or behavioural parameters of the (prenatally stressed) offspring can also be used to validate the stress paradigm. In accordance with previous studies, prenatal stress did not affect birth weight, whereas body weight development was reduced (Keshet and Weinstock, 1995; Buhl et al., 2007; Leonhardt et al., 2007; Darnaudery and Maccari, 2008). Since stressed dams display an increase in active nursing (ABN), future studies are required to assess whether the decreased weight gain in the prenatally stressed pups is due to stress-induced changes in the nutritional content of the milk or deficits in the physical development of the offspring.

As previously shown in rats bred for high (HAB) or low (LAB) anxiety, assessment of ultrasonic calls is a well-validated anxiety parameter at an early stage of life (Landgraf and Wigger, 2002). We could show that prenatally stressed pups showed an increased number of USV when separated from the mothers on postnatal day (PND) 8, indicative of increased anxiety, providing an additional validation of the chronic pregnancy stress procedure.

Pregnancy stress prevents the lactation-associated basal hypercorticism

It has been previously reported that lactation is associated with an attenuated rise to acute stressors, of ACTH and CORT (Neumann et al., 1998b), which could only be partly confirmed in the present study. Importantly, we could observe that the basal hypercorticism observed

during lactation in rodents and humans (Stern et al., 1973; Neumann et al., 1998b; Heinrichs et al., 2001; Lightman et al., 2001; Neumann, 2008), could be prevented by pregnancy stress. This is an interesting finding as enlarged adrenals were also found in stressed lactating dams, which suggests that stress causes adrenal insufficiency in dams. Similar findings have previously been described following chronic psycho-social stress exposure in male mice and appear to be a particular consequence of social-based chronic stressors (Reber et al., 2007; Reber, 2011). Traditionally, increased CORT levels have been associated with chronic stress exposure (for review see Blanchard et al., 1993), but it is becoming apparent that chronic stress can also result in decreased adrenal function (Bremmer et al., 2007; Reber and Neumann, 2008). Furthermore, this hypothesized adrenal insufficiency in the stressed dams mirrors those from a recent study in humans showing decreased awakening cortisol levels in women, who develop postnatal depression (Taylor et al., 2009). These data could suggest that chronic pregnancy stress results in a similar outcome as post-traumatic stress disorder or chronic fatigue syndrome, which are associated with a state of hypocorticism. Interestingly, the latter is also a particularly prevalent symptom of postpartum depression (Kammerer et al., 2009; Taylor et al., 2009). However, chronic stress exposure might also alter plasma CBG levels, which would, in turn, affect plasma levels of free CORT, which will be assessed in future studies. Taken together, these results suggest that low basal glucocorticoid plasma levels may represent a biomarker for postpartum mood and anxiety disorders, and more research is warranted to investigate this possibility.

Pregnancy stress does not alter AVP or CRH mRNA but prevents the peripartum rise in hypothalamic OXT mRNA

The elevation in basal CORT levels in lactation is believed to be due, at least in part, to alterations in the expression of relevant hypothalamic neuropeptides, such as AVP and CRH (Brunton et al., 2008; Slattery and Neumann, 2008; Hillerer et al., 2011a). In the present study, AVP mRNA was not altered by stress or pregnancy. Although increased AVP expression in parvocellular PVN neurons during lactation has previously been demonstrated (Walker et al., 2001b), the current data were assessed at the end of pregnancy and within the whole PVN, which could explain this apparent discrepancy. In contrast, while a decrease in CRH mRNA expression was observed in the peripartum period compared with virgins as previously shown (Fischer et al., 1995; Windle et al., 1997; Shanks et al., 1999; Walker et al., 2001b), there was no effect of chronic pregnancy stress on this adaptation. Taken together with the neuroendocrine data, these findings suggest that alterations in CRH or AVP neuronal activity are unlikely to mediate the chronic stress-induced difference in basal CORT levels in the dams; although receptor binding and release patterns at the level of the pituitary would have to be assessed to confirm this. Therefore, future studies will assess whether changes at the level of the adrenal glands could mediate the stress-induced reduction of peripartum-associated hyper-CORT.

Increased activity of the brain OXT system from the end of pregnancy into lactation is reflected by increased expression of hypothalamic OXT mRNA and its receptor (Zingg et al., 1995; Windle et al., 1997; Bosch and Neumann, 2008; Figueira et al., 2008; Slattery and Neumann, 2008), and intracerebral OXT release triggered by reproductive stimuli (Neumann and Landgraf, 1989; Neumann et al., 1993; Neumann, 2007). Therefore, we assessed OXT mRNA and could demonstrate that pregnancy stress prevents the peripartum-induced rise

within the PVN. Interestingly, in a recent study, first-time mothers who had low attachment ratings prior to birth also displayed lower activation of the reward circuitry and lower plasma OXT levels when interacting with their infants (Strathearn et al., 2009). Furthermore, healthy women who had experienced childhood trauma were shown to have lower levels of OXT in cerebrospinal fluid (CSF) compared with controls and also displayed the lowest attachment to their infants (Heim et al., 2009). Although other systems are likely to be involved, these studies suggest that a certain activity of the brain OXT system, which in humans can only be estimated by CSF concentrations, is required to mediate the rewarding aspects of mother-infant relations. The importance of OXT in the regulation of postpartum emotionality has recently been emphasized by the demonstration of a correlation between low plasma OXT concentrations during pregnancy and the development of postpartum depression (Skrundz et al., 2011).

Pregnancy stress alters maternal behaviour and prevents lactation-associated anxiolysis

Chronic pregnancy stress led to increased ABN in the light phase from early to mid-lactation, which was most prominent during early lactation. Despite the increase of ABN in the light phase, we did not find such differences between stressed and non-stressed dams in the dark phase, which is likely due to the low level of nursing in the active phase. We have previously demonstrated the relevance of ABN, to both the mother (see below) and the offspring, in a number of studies. For example, when offspring of high-anxiety related behaviour (HAB) dams were subjected to 3 h MS for the first 14 days of life, consequently reducing their abnormally high amount of nursing received, they display reduced anxiety in adulthood (Neumann et al., 2005a). Moreover, a similar trend towards reduced anxiety in adulthood

was observed when HAB mice, which also receive increased levels of ABN, were cross-fostered to less attentive low anxiety-related behaviour (LAB) dams (Kessler et al., 2011). In contrast, and as shown in out-bred rats, LAB rats subjected to the same MS paradigm (further reducing their lower ABN received) displayed an increased anxiety level in adulthood (Neumann et al., 2005a; Bosch, 2011). Prenatally stressed offspring in the present study were also shown to exhibit higher levels of anxiety in adulthood (Hillerer, unpublished observations). Therefore, it appears that an optimal level of ABN has to be provided for the most favourable development of the offspring. Although differences in licking behaviour have previously been described following gestational stress (Champagne and Meaney, 2006), we did not observe any pregnancy stress-induced differences in this behaviour (data not shown). However, in addition to the different timing of stress exposure, the previous study was performed in out-bred Long-Evans rats, and we have previously demonstrated that Wistar rats (as used in the current study) do not perform this behaviour as frequently (Neumann et al., 2005b; Bosch, 2011). Therefore, alteration in active nursing is a more reliable indicator of a stress-induced alteration in maternal care in our laboratory (Bosch and Neumann, 2008).

While OXT is required for the onset of maternal behaviour, it is not required for its maintenance (Russell et al., 2001), which may partly explain the apparent discrepancy between the increased maternal behaviour and reduced OXT mRNA found in the stressed dams of the current study. Rather, the reduced activity of the brain OXT system is likely to be linked to the elevated anxiety found in pregnancy-stressed compared with non-stressed dams in mid-lactation as it has previously been shown that central application of an OXT-R antagonist increases anxiety in lactating, but not virgin or male, rats (Neumann et al., 2000a). Interestingly, the anxiogenic effect of pregnancy stress can perhaps explain their

altered patterns of maternal behaviour, as high anxiety has repeatedly been linked to a high levels of maternal behaviour, including increased ABN (Maestripieri and D'Amato, 1991; Champagne and Meaney, 2006; Bosch and Neumann, 2008; Bridges, 2008; Braw et al., 2009; Hillerer et al., 2011a; Kessler et al., 2011). This correlation has been observed in both mice and rats, and suggests that it is a, biologically-relevant, evolutionary adaptation (Slattery et al., 2005; Heim et al., 2009). While one major feature of postpartum depression is a significant lack of child attachment, and impaired, or even lack of, maternal care has been reported in women suffering from postpartum mood disorders (Lyons-Ruth et al., 1986; Bifulco et al., 2004), it has been shown that highly-anxious mothers tend to ruminate over the health and well-being over their babies (helicopter parenting) (Bosch and Neumann, 2008; Bridges, 2008).

Pregnancy stress and depression-like behaviour

Noradrenergic input from the brainstem, as well as adrenergic receptor expression in the PVN, is down-regulated peripartum, which contributes to the decreased stress responsiveness (Douglas, 2005). These changes may also contribute to the observed decrease in climbing behaviour in the FST compared with virgins (Hillerer, unpublished data) as climbing has been shown to be mediated by catecholamines (Cryan and Slattery, 2010). Therefore, we determined the effect of acute imipramine, as it has been shown (together with its metabolite desimipramine) to act predominantly on the noradrenergic system and as such selectively increase climbing behaviour (Cryan et al., 2002; Slattery et al., 2005), on stress-coping behaviour in stressed and non-stressed dams. As expected, imipramine reduced the passive behaviour in the non-stressed dams, but tended towards the opposite

effect in the stressed dams. This suggests that stress exposure may not only have prevented the physiological attenuation of the noradrenergic system established in the peripartum period (Douglas, 2005; Douglas et al., 2005), but that it may alter the response to increased synaptic noradrenaline. Future studies will examine whether the attenuated noradrenaline release within the PVN and the decreased receptor expression is prevented by pregnancy stress. In contrast, depression-like behaviour was not altered between vehicle-treated non-stressed and stressed dams suggesting that pregnancy stress does not lead to a depressive-like phenotype.

CONCLUSIONS

While it is unlikely that a rodent model of a specific postpartum mood or anxiety disorder is achievable, by simultaneously examining numerous peripartum adaptations, we could demonstrate the concerted impact of chronic stress exposure on these systems. Prevention of the basal peripartum-associated increases in plasma CORT and OXT system activity by pregnancy stress indicates two alterations that may increase the risk of postpartum mood and anxiety disorders. These changes are more likely to be linked to postpartum anxiety, given the higher anxiety-related behaviour and maternal care observed following stress. However, further studies are required, both basic and clinical, to further assess whether the stress-induced prevention of peripartum adaptations represent, at least partially, the underlying causes of postpartum mood and anxiety disorders, and if they potentially provide markers to assess the likelihood of a mother developing such a disorder.

Chapter 3

Hippocampal neurogenesis is differentially regulated under basal and chronic stress conditions in male and female rats

Author's contribution:

Hillerer: Study design, performing of experiments, analyzing data, writing the manuscript

Neumann: Study design

Couillard-Despres: Establishment of staining, contribution to manuscript writing

Aigner: Study design, contribution to manuscript writing

Slattery: Study design, performing experiments, contribution to manuscript writing

[adapted from: Hillerer K.M., Neumann I.D., Couillard-Despres S., Aigner L., Slattery D.A. Hippocampal neurogenesis is differentially regulated under basal and chronic stress conditions in male and female rats. Hippocampus; in preparation]

ABSTRACT

Sex differences in both basal and stress-induced hippocampal neurogenesis processes have been reported in the literature. However, studies directly comparing basal and stress-induced sex differences on multiple neurogenesis processes are lacking to date. Therefore, the aim of the present study was to directly compare cell proliferation and survival, neuronal and astroglial differentiation and stem cells quiescence in males and females under both basal and chronic stress conditions (12 days of 2 h RS). In addition, CORT levels and spatial working memory were assessed. Under basal conditions, only the number of immature neurons was different between males and females. In contrast, chronic stress resulted in a number of sex-specific alterations. Thus, stress exposure reduced cell proliferation in males, which was reflected by an increase in stem cell quiescence, while it did not alter either parameter in females. In contrast, stress exposure decreased cell survival in females only. Analysis of astroglial and neuronal differentiation patterns revealed that chronic stress specifically diminished the number of mature neurons in females, with no effect in males. Despite the observed sex differences in adult hippocampal neurogenesis, spatial working memory was unchanged in either sex. Basal CORT levels were higher in females, whereas exposure to chronic stress did not affect basal plasma CORT levels in either sex across the stress period. This is the first direct comparison of sex-dependent and chronic stress-induced changes in adult hippocampal neurogenesis, which will lead to a better understanding of sex differences in neurogenesis processes.

INTRODUCTION

It is now a well-established fact that new cells can be produced throughout the lifespan in the brain of different species like mice (Kempermann et al., 1997), rats (Cameron and Gould, 1994), tree shrews (Gould et al., 1997), monkeys (Uno et al., 1989) and humans (Eriksson et al., 1998). This effect, termed neurogenesis, occurs predominantly in two brain regions namely the SGZ of the DG and the SVZ. The DG develops during gestation, and continuously undergoes remodelling throughout the lifespan of the organism, suggesting that the production of new neuronal cells plays an important role in the hippocampal function. Thus, adult hippocampal neurogenesis plays an important role in learning and memory and *vice versa* (for reviews see Leuner et al., 2006; Koehl and Abrous, 2011), but moreover, there is increasing evidence that it is also involved in the pathogenesis of stress-related disorders, such as depression (for reviews see Czeh and Lucassen, 2007; Sahay and Hen, 2007; Lucassen et al., 2010). Although adult hippocampal neurogenesis is a species-wide phenomenon, sex differences exist in all aspects studied to date (for review see Pawluski et al., 2009). Accordingly, males and females differ in their capacity to produce new hippocampal neurons and females have been shown to express a greater number of mossy fibre synapses in the CA3 region compared to males (Madeira et al., 1991). Additionally, males and females have differing mechanisms that regulate granule neuron production, with females producing more granule cells than males (Handa et al., 1994), but also exhibiting a higher degeneration rate than males (Tanapat et al., 1999). These naturally occurring sex differences may be due to the levels of different circulating hormones during the development and adulthood (McEwen et al., 1995). Ovarian hormone levels, in particular, have been shown to be of importance in the regulation of adult hippocampal neurogenesis.

Thus, they affect the number of hippocampal synapses (Woolley and McEwen, 1992), the strength of hippocampal long-term-potentiation (LTP) and modulate hippocampal-dependent learning (Daniel et al., 1997).

In addition to these sex differences, hippocampal neurogenesis is greatly influenced by external factors, such as environmental (both positive and negative; for reviews see Pham et al., 2002; Leuner and Gould, 2010) and pharmacological manipulations (for reviews see Banasr and Duman, 2007; Schmidt and Duman, 2007; Lucassen et al., 2010). Thus, stress is an important factor that affects all aspects of adult neurogenesis and it is well-known that males and females exhibit different stress sensitivity and coping mechanisms (Bowman et al., 2001; Wolf et al., 2001; Dalla et al., 2008). In contrast to males, who habituate to stressful situations, females show a longer and more robust rise in CORT levels (Galea et al., 1997; Falconer and Galea, 2003). The sex differences in HPA axis activity seem to be of particular importance, given the fact that increased glucocorticoid levels are believed to be one of the primary mediators underlying the detrimental effects of stress on adult hippocampal neurogenesis. Therefore, given these stress-coping and stress-response differences, it is not surprising that they are mirrored in a sex-dependent manner in neurogenesis patterns. The majority of studies performed in male rodents, to date, have demonstrated that chronic stress diminishes cell proliferation in the DG (Malberg and Duman, 2003; Westenbroek et al., 2004; Shors et al., 2007; Silva et al., 2008) without affecting cell survival (Joels et al., 2004), induces apical dendritic atrophy of CA3 pyramidal neurons (Uno et al., 1989; Magarinos and McEwen, 1995; Galea et al., 1997) and reduces the number of branching points in males (Galea et al., 1997). Fewer studies have been performed in female rodents, with the consensus revealing that chronic stress does not seem to affect cell proliferation or apical dendrite morphology (Uno et al., 1989; Galea et al.,

1997; Westenbroek et al., 2004; Shors et al., 2007). However, the literature assessing the effects of chronic stress exposure on cell survival in females is controversial with studies showing either an increase (Westenbroek et al., 2004), or a decrease (Kuipers et al., 2006) in the number of surviving cells.

Despite all the studies assessing sex differences on cell proliferation, cell survival and dendritic morphology after chronic stress exposure, a study examining how chronic stress affects cell fate and quiescence of stem cells is missing so far. Moreover, the studies performed to date have focussed on either male or female rodents, what further complicates the comparison of sex-related differences in neurogenesis.

Therefore, the aim of our study was to determine not only basal sex differences in cell proliferation, cell survival, cell fate and quiescence, but moreover to assess the impact that chronic stress exposure had on these parameters.

MATERIALS AND METHODS

Animals

Female (200-250g) and male (250-300g) Wistar rats, 9-10 weeks old (Charles River, Sulzfeld, Germany) were housed in same-sex groups of four in standard polycarbonate rat cages and allowed to habituate for at least seven days. After habituation, all animals were single-housed for 7 days before the experimental procedures commenced. All rats were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00h, 22°C ± 1°C, 60 55 ± 5% humidity) and had free access to water and standard rat diet. All experimental

procedures were performed between 08:00 – 12:00 A.M., approved by the Committee on Animal Health and Care of the local government of the Oberpfalz, and complied with international guidelines on ethical use of animals.

Chronic stress procedure

Animals of stressed groups were subjected to 2 h RS, which is an effective stressor in male and female rats (Hillerer et al., 2011a; Hillerer et al., 2011b) for 12 consecutive days between 10:00 - 12:00 A.M.. Each rat was placed in a plexiglass column with ventilation holes (12 cm diameter). Non-stressed controls were single-housed and left undisturbed in their home cages in the same room. The body weight of each animal was recorded daily from the beginning of the stress procedure until the day of sacrifice.

Experiments 1 and 2: Assessment of hippocampal cell proliferation, cell survival, cell fate and quiescence under basal and chronic stress conditions

BrdU labelling

To examine the proliferation of precursor cells, rats were injected with 5-Bromo-2'-desoxyuridine (BrdU; 50mg/kg, i.p.) once, on the last day of stress, immediately after removal from the restraint tubes, i.e at 12:00 A.M.. 24 h after BrdU injection animals were deeply anesthetized with ketamine/ xylazine (90-120 mg/kg ketamine and 6-8mg /kg xylazine diluted in 0.9% NaCl), intra-cardiacally perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. 24 h have been reported to be sufficient for newborn cells to complete one cell cycle (Takahashi et al., 1992).

To trace the survival and fate of recently born cells, rats received daily injections of BrdU (50mg/kg, i.p.) during the first four days of stress exposure (day 1-day 4) as described above. 16 days after the last BrdU injection (day 20) rats were anesthetized and perfused as described above. BrdU (Sigma/Aldrich) was freshly prepared in 0.9% NaCl solution to a dilution of 20mg/ml on each injection day.

(For schematic representation of the temporal design of the study see *Figure 7* of the result section)

Histological procedures

Immediately after the perfusion brains were weighed to determine absolute and relative brain weights, post-fixed in 4% paraformaldehyde at 4°C overnight, before transfer to 30% sucrose, 0.1 M sodium phosphate solution (pH 7.4) in sterile water for at least one week. Sagital brain sections (40µm) were prepared using a sliding microtome on dry ice and subsequently stored at 4°C in a cryoprotection solution (glycerol, ethylene glycol and 0.1 M phosphate buffer, pH 7.4, at a ratio of 1:1:2 by volume; (Kandasamy et al., 2010)). Immunostaining of BrdU-labelled cells was performed on free-floating sections using the diaminobenzidine (DAB) peroxiodase method. Briefly, brain sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min. For DNA denaturation, sections were incubated for 2 h in 50% formaldehyde/2 X saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, rinsed for 5 min in 2 X SSC, incubated in 2M HCl for 30 min at 37°C and washed for 10min in 0.1M boric acid, pH 8.5. Thereafter, sections were incubated in Fish skin gelatine buffer (FSGB) for 1 h, followed by incubation with the primary rat α- BrdU antibody (1:500, Oxford Biotechnology, Oxford, UK)

in FSGB overnight at 4°C. The next day, the sections were incubated with biotinylated secondary donkey α-rat antibody (1:500, Molecular Probes), followed by the avidin-biotin-peroxidase complex reaction (1 h; Vectastain elite ABC kit; Vector Laboratories, Burlingame, CA (Kandasamy et al., 2010)). Thereafter, the signal was visualized using DAB (25mg/ml in water with 0.01% H_2O_2, 0.04 $NiCl_2$). Stained slides were mounted on microscopic slides, washed with NeoClear (Merck) and cover-slipped with NeoMount (Merck). Immunostaining of DCX-labelled cells was performed on free-floating sections as described above (antibodies used: 1° AB goat α DCX , 1:250 in FSGB (Santa Cruz Biotechology; Santa Cruz, CA); 2° AB biotinylated donkey α goat (Molecular Probes); (Kandasamy et al., 2010)). Triple-immunfluorescence for BrdU/ GFAP/ NeuN was performed using a standardised protocol (Kandasamy et al., 2010). Briefly, free floating brain sections were incubated in 50% formaldehyde/2 X saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) at 65°c for 1 h, rinsed for 5 min in 2 X SSC, incubated in 2M HCl for 30 min at 37°C and washed for 10 min in 0.1M boric acid, pH 8.5. Thereafter, sections were washed four times in TBS for 5 min, before they were incubated with FSGB for 30 min, followed by incubation with the antibody mix (BrdU α rat, 1:500,Oxford Biotechnology, Oxford, UK; GFAP α guinea pig, 1:500, Progen; NeuN α mouse, 1:500, Chemicon) in FSGB for 48h at 4°C. 48h later, the sections were incubated overnight with the secondary antibody mix (donkey α rat conjugated with rhodamine red, 1.500; donkey α guinea pig conjugated with IgG Cy5, 1:500; donkey α mouse conjugated with Alexa Fluor 488, 1.500) in FSGB, shaking in the dark. Stained slides were mounted on microscopic slides and cover-slipped with Prolong- Antifade (Molecular Probes). Triple-immunfluorescence for BrdU/PCNA/ SOX2 was performed as described above (used antibodies: 1°AB: BrdU α rat, 1:500, Oxford Biotechnology, Oxford, UK; PCNA α mouse, 1.500, Santa Cruz Biotechnology, Santa Cruz, CA; SOX2 α goat , 1:500, Santa Cruz

Biotechnology, Santa Cruz, CA; 2°AB: donkey α rat conjugated with rhodamine red, 1.500; donkey α goat conjugated with Alexa Fluor 660, 1:500; donkey α mouse conjugated with Alexa Fluor 488, 1:500; (Kandasamy et al., 2010)).

Stereology

To determine the number of BrdU-positive cells, every sixth section (240µm interval) of the right hemisphere was examined for BrdU- positive cells throughout the rostral caudal extent of the granule cell layer and the adjacent SGZ. Cells were counted regardless of shape or size under 10X magnification. We used a semi-automatic stereology system (Stereoinvestigator, MicroBrightField) and a 5X objective to trace a defined area of the DG / SGZ. The defined area was used to calculate the number of BrdU-positive cells per $100m^2$ of the DG.

Confocal analysis

All morphological analyses were performed by an experimenter blind to the group. To determine the frequency of neuronal differentiation of newborn cells, a series was examined using a grid confocal laser microscope (Olympus XI81) using every sixth section (240µm interval). Z-stacks were built using the Volocity Software (Perkin Elmer). 50 BrdU-positive labelled cells per animal were analysed for neuronal differentiation. BrdU-positive cells were counted as solely BrdU-positive (newborn cells), BrdU/ NeuN (newborn neurons) double-positive cells and BrdU/GFAP (newborn astrocytes) double-positive cells. The same procedure was performed to determine the frequency of quiescent stem cells. BrdU-positive cells were counted as solely BrdU-positive (newborn cells), BrdU-positive/PCNA-

negative/SOX2-positive (quiescent stem cells) and BrdU-positive/ PCNA-positive/ SOX2-positive (proliferating stem cells)

Experiment 3: Effect of stress exposure on plasma CORT levels

As CORT levels have been shown to be an important regulator of neurogenesis (Magarinos and McEwen, 1995; Galea et al., 2008; Barha et al., 2011), we measured CORT levels under basal and stress conditions. Therefore, a jugular vein surgery was performed as previously described (Neumann et al., 1998b; Bosch et al., 2007b; Hillerer et al., 2011b). Briefly, the jugular vein was exposed by blunt dissection, then a catheter consisting of silicon tubing (Dow Corning Corp., Midland MI, USA) and PE-50 polyethylene tubing was inserted approximately 3 cm into the vessel through an incision in a cardiac direction and exteriorized at the neck of the animal. The catheter was filled with sterile saline containing gentamicin (30,000IU/ml; Centravet, Bad Bentheim, Germany). Five days after surgery, on the first day of chronic stress procedure, i.e day 1, day 3 and day 5 at 07:30, the catheter was attached to an extension tube connected to a 1-ml plastic syringe filled with sterilized heparinised 0.9% saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany). Each rat was then left undisturbed for 2 h. Two basal samples (basal sample one: 0.6ml and basal sample two: 0.2ml) were taken 30 min apart and were used to calculate the mean basal concentrations for CORT. The percentage CORT concentration as shown in Fig. 11, was calculated using the mean CORT concentration of male non-stressed animals on day 1 (397.6 ng/ml). After the sampling of basal sample 2 rats were placed into the restraint tubes. After 2 h rats were removed from the restraint tubes and put back in their home-cage. All blood samples were immediately replaced with the same volume of intravenous sterile 0.9% saline. All blood

samples were collected on ice in EDTA-tubes containing aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) and analysed for CORT using a commercially available ELISA kit.

Experiment 4: Effect of stress exposure on spatial memory in the Y-maze

As cognition has been shown to be closely linked to neuroplasticity (for review see Galea et al., 2008), we assessed immediate spatial working memory by recording spontaneous alterations in a single session in the Y-maze. The Y-maze consisted of three equally spaced arms conducted to each other at one end. Each arm was 50 cm long and 16 cm wide with 40 cm high walls. The test was performed one day after the end of stress, in animals that were also used for the analysis of cell survival, differentiation and quiescence (see also *Figure 7B*) (one female outlier was excluded from analysis). Each rat (naive to the maze) was placed at the end of one arm, the head facing the walls and allowed to freely explore the maze during a 10 min session. A camera above the maze enabled assessment of the number and the sequence of arm entries by an observer blind to group. An arm entry was scored when all four paws crossed into the arm. The percentage of alternation was calculated by the formula: number of alterations / maximal theoretical number of alternations X 100, where the alternation was defined as consecutive entries into three different arms.

Statistical analysis

All numerical data are expressed as the mean ± SEM and statistically analysed using a Mann Whitney *U* test, or a one- or two- way analysis of variance (ANOVA) with or without repeated measures, as appropriate. Any statistical differences, which were set at $p< 0.05$,

were further analysed using a Fisher's *post-hoc* test. Statistical analyses were performed using SPSS for Windows (version 16; SPSS Inc, Chicago, IL, USA).

RESULTS

Basal and chronic stress-induced sex differences in cell proliferation

To assess sex differences on cell proliferation under both basal and chronic stress conditions, animals were given a single BrdU injection after 12 d RS and perfused 24 h later (see *Figure 7A*). Two-way ANOVA revealed a significant interaction between chronic stress exposure and sex ($F_{1,23}$=8.504; p=0.008). *Post-hoc* analyses revealed a decrease in cell proliferation in stressed males compared with non-stressed controls (p<0.01; Fig. 8A) and a lower number of proliferating cells in stressed males compared to the respective female group (p<0.05; Fig. 8A). However, no differences in basal cell proliferation between males and females were observed (p>0.05; Fig 8A).

Basal and chronic stress-induced sex differences in stem cell quiescence

We next examined sex differences and chronic stress-effects on the number of quiescent and proliferating stem cells. Therefore, animals received BrdU injections on the first 4 days of stress (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 7B*). The number of quiescent stem cells (BrdU positive, SOX2 positive, PCNA negative) was found to be independent of sex ($F_{1,20}$=2.032; p=0.914) and stress ($F_{1,20}$=3.872; p=0.063).

However, separate analysis revealed an increase in the number of quiescent stem cells in the DG of stressed males compared with the respective non-stressed group (Mann Whitney *U*; p=0.01; Fig. 8B). Furthermore, there was no effect of sex ($F_{1,20}$=3.876; p=0.062) or stress ($F_{1,20}$=3.980; p=0.063) on the number of proliferating stem cells (BrdU positive, SOX2 positive, PCNA positive; Fig. 8C). However, separate analysis revealed a reduction in proliferating stem cells in males exposed to chronic stress, when compared to control males (Mann Whitney *U*; p=0.004; Fig. 8C).

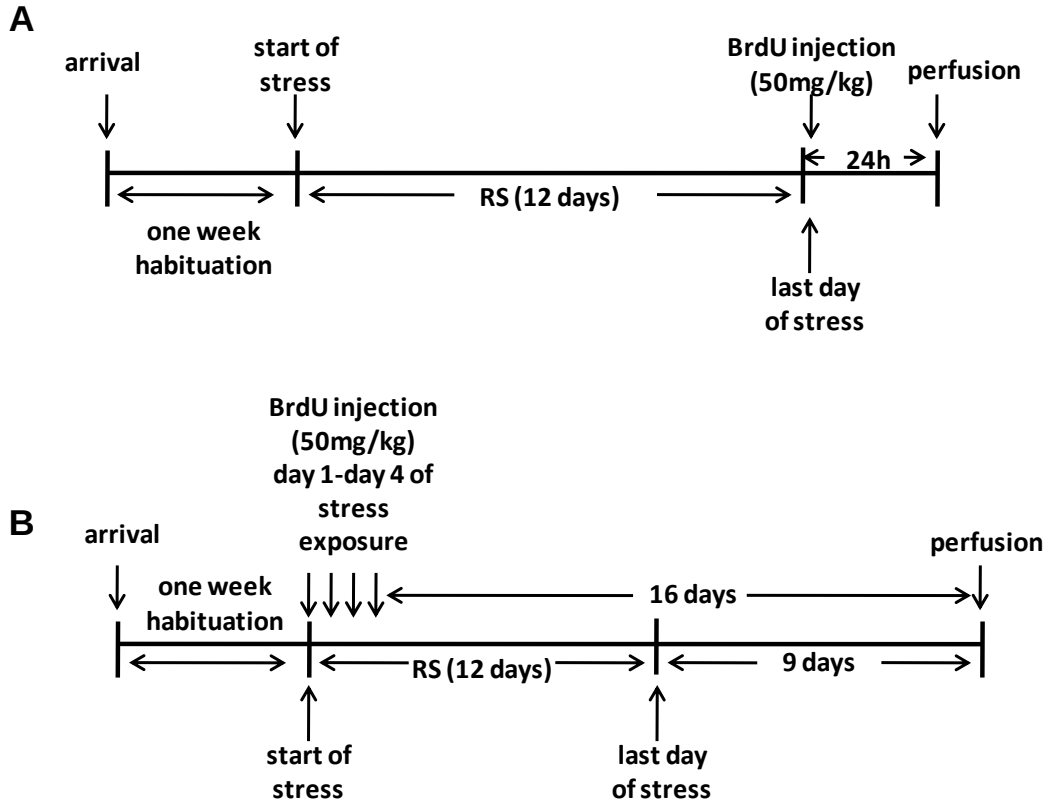

Figure 7: Schematic representation of the temporal designs used in the present studies

Temporal designs used to assess A) cell proliferation, immature neuron production (DCX positive cells) and B) cell survival, neuronal/ astroglial differentiation, quiescent/ proliferating stem cells and spatial working memory. After one week of habituation, animals were exposed to 2 h of RS on 12 consecutive days. They received either A) a single BrdU injection (50mg/kg; i.p.) on the last day of stress or B) four BrdU injections (50mg/kg; i.p.) on 4 consecutive days (day 1- day 4 of chronic stress exposure). Animals were intra-cardiacally perfused either A) 24 h after or B) 16 days after the last BrdU injection.

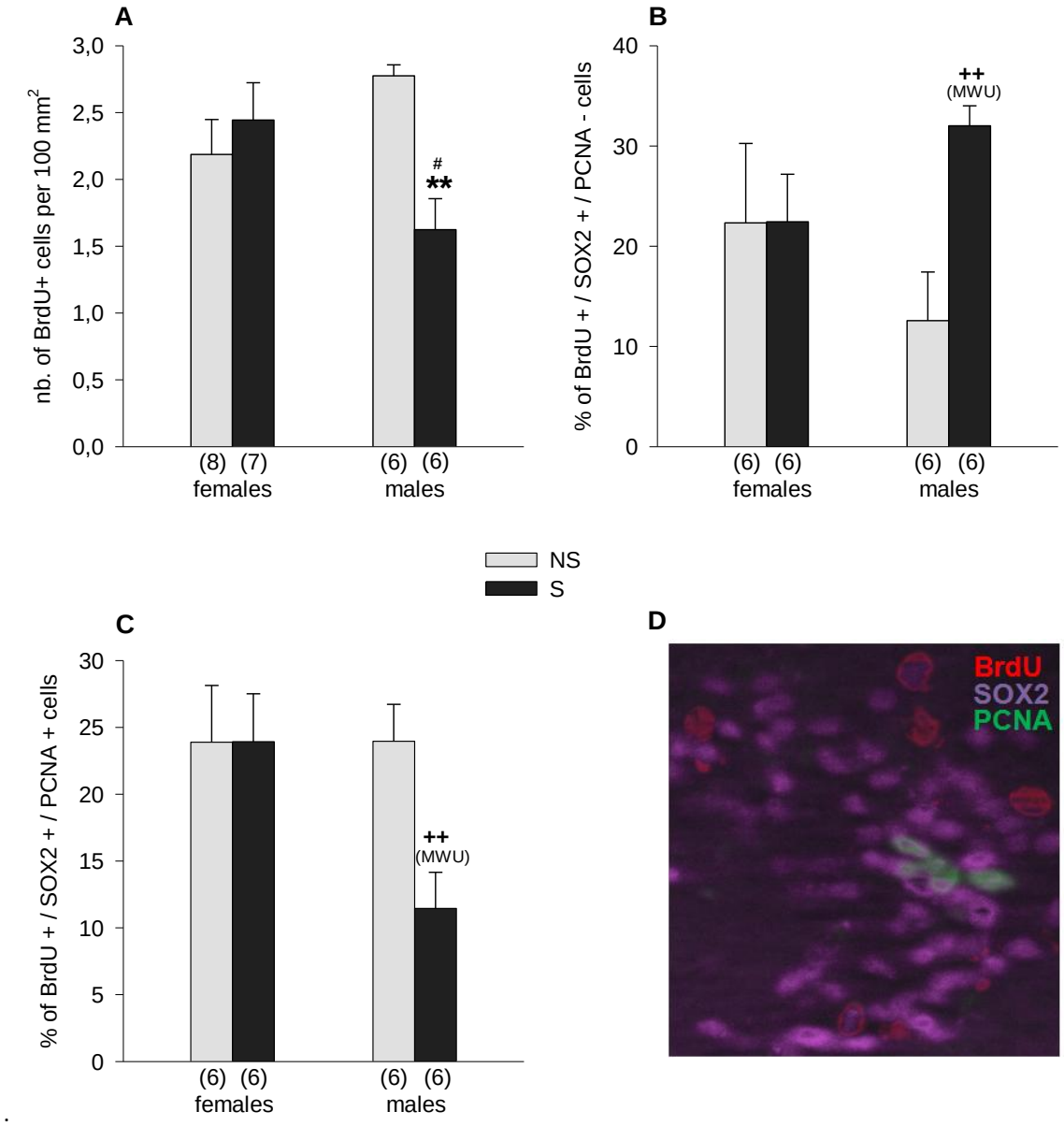

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Figure 8: Effect of sex and chronic stress on cell proliferation and stem cell quiescence in the DG

The total number of proliferating cells (A), the percentage of quiescent stem cells (B) and percentage of proliferating stem cells (C) was assessed in the DG of the hippocampus in males and females under basal (grey bars) or chronic stress (black bars) conditions. A) Chronic stress led to a lower number of proliferating cells in males *vs.* their respective non-stressed group and *vs.* the stressed female group; no basal sex differences were observed. B) Analysis of the number of BrdU positive, SOX2 positive and PCNA negative cells, indicative for stem cell quiescence revealed no basal differences between males and females, however, the percentage of quiescent stem cells was increased in stressed *vs*. non-stressed males. C) Chronic stress led to a reduction in proliferating stem cells (BrdU positive, SOX2 positive, PCNA positive) in males, but not females; no basal sex differences were observed. D) Representative image of the triple-immunfluorescent image (male non-stressed animal) with antibodies against BrdU (red), SOX2 (violet) and PCNA (green). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. **, p<0.01 *vs.* respective non-stressed group; #, p<0.05 *vs.* respective female group; ++, p<0.01 *vs.* respective non-stressed group using a Mann Whitney *U* test (MWU). NS, non-stressed; S, stressed

Basal and chronic stress-induced sex differences on immature neuron production

To examine sex-occurring and stress-dependent differences in the number of immature neurons between males and females, animals were given a single BrdU injection and perfused 24 h later (see *Figure 7A*). There was a significant effect of sex ($F_{1,24}$=12.252; p=0.02), but not stress ($F_{1,24}$=0.275; p=0.605) on the number of DCX positive cells in the DG. *Post-hoc* analyses revealed a higher number of immature neurons under both basal and chronic stress conditions in males compared to the respective female group (p<0.05; Fig. 9A).

Basal and chronic stress-induced sex differences on cell survival

We next assessed the effect of sex and chronic stress on cell survival in the DG. Therefore, animals received BrdU injections on the first 4 days of stress (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 7B*). Two-way ANOVA revealed effects of stress ($F_{1,21}$=4.867; p=0.039) and sex ($F_{1,21}$=13.485; p=0.001) on cell survival in the DG. *Post-hoc* analyses revealed a lower cell survival in males compared to females under basal conditions (p<0.001; Fig. 9B), as well as a reduction in the number of surviving cells in females after chronic stress exposure (p<0.01; Fig. 9B). However, no effect of stress on cell survival was observed in males (p>0.05; Fig. 9B).

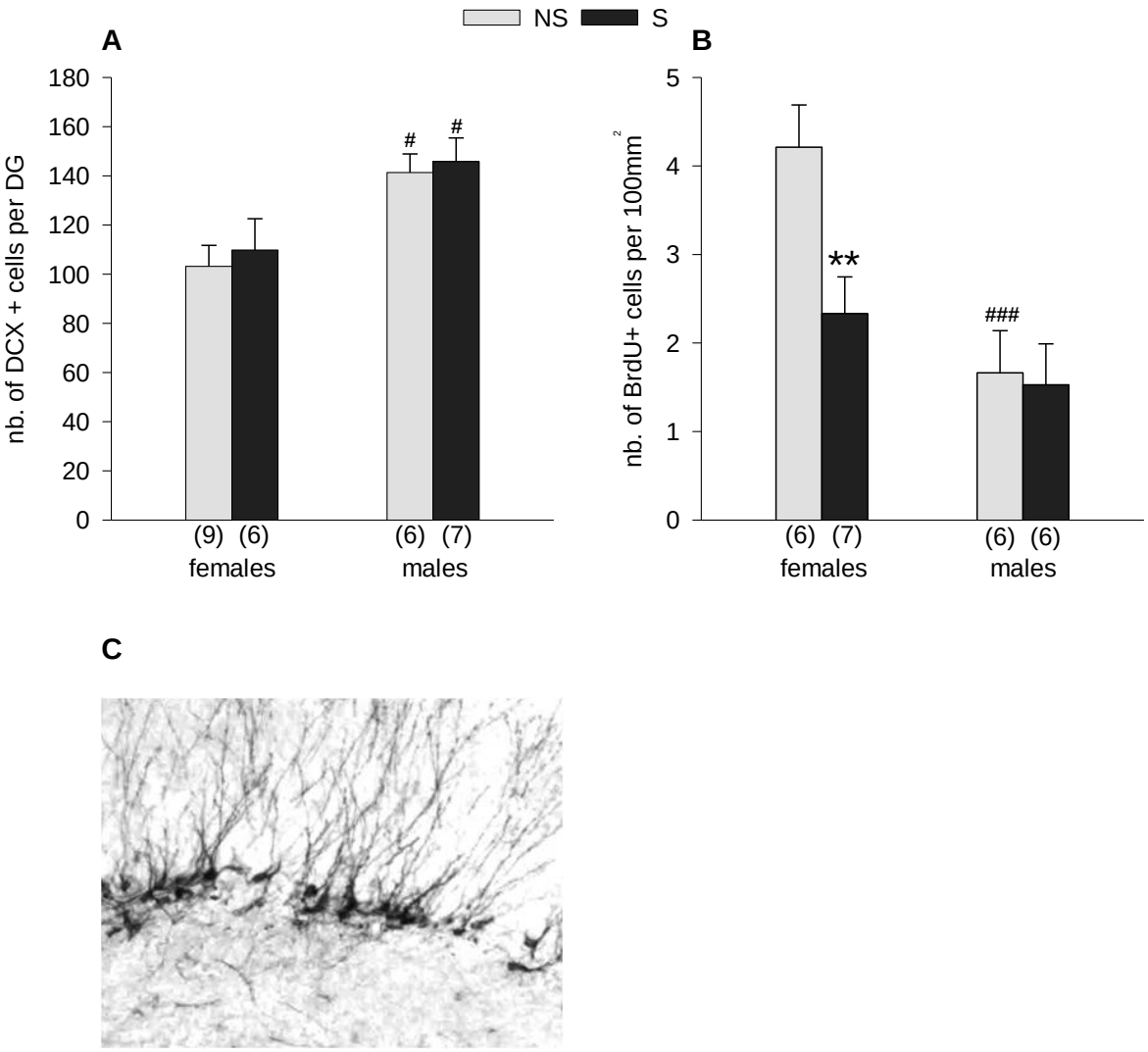

Figure 9: Effect of sex and chronic stress on immature neuron production and survival of cells

The number of immature neurons (DCX+ cells) (A) and the number of surviving cells (B) was assessed in the DG of the hippocampus in females and males under basal (grey bars) or chronic stress (black bars) conditions. A) Males expressed higher numbers of immature neurons under basal and chronic stress conditions compared with females. B) Under basal conditions, males had a lower number of surviving cells compared to females; whereas chronic stress diminished the number of surviving cells in females, but had no effect in males. C) Representative image (male non-stressed animal) of a DAB-immunostaining against DCX. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. **, $p<0.01$ *vs.* respective non-stressed group; #, $p<0.05$ *vs.* respective female group; ###, $p<0.001$ *vs.* respective female group. NS, non-stressed; S, stressed

Basal and chronic stress-induced sex differences on neuronal and astroglial differentiation

To evaluate if males and females vary in their astroglial and neuronal differentiation patterns, animals received BrdU injections on the first 4 days of stress (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 7B*). No effect of sex ($F_{1,21}$=0.09; p=0.768) or stress ($F_{1,21}$=2.118; p=0.160) on neuronal differentiation was observed (Fig 10A). However, separate analysis revealed a reduction in the percentage of neuronal differentiation in stressed females compared with the respective non-stressed group (Mann Whitney *U*; p=0.022; Fig. 10A). Astroglial differentiation was shown to be independent of stress ($F_{1,21}$=0.000; p=0.991) and sex ($F_{1,21}$=1.795; p=0.195) (Fig. 10B).

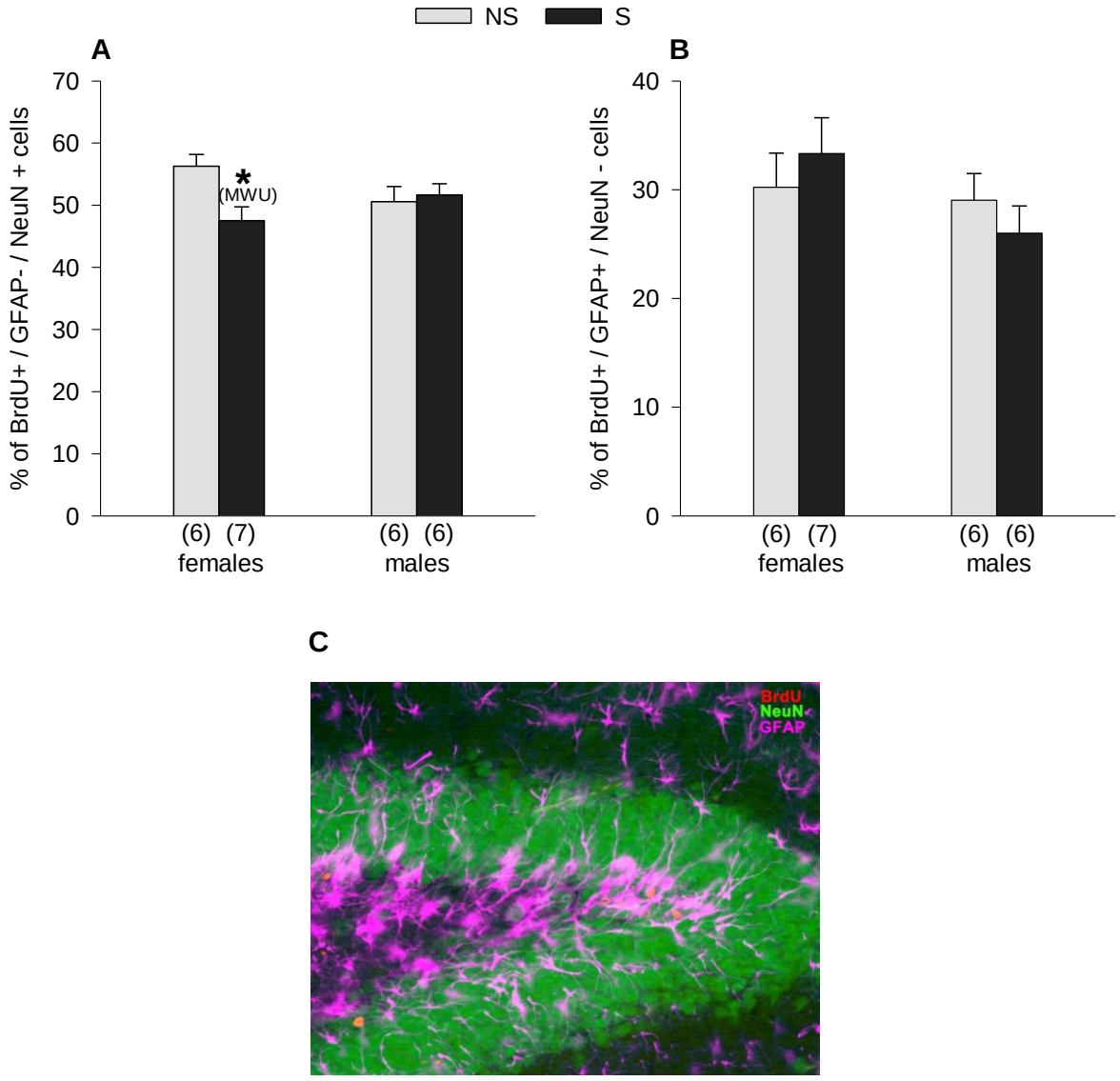

Figure 10: Effect of sex and chronic stress on differentiation patterns

The percentage of cells differentiating into neurons (BrdU positive, GFAP negative and NeuN positive cells) (A) and astroglial cells (BrdU positive, GFAP positive, NeuN negative cells) (B) was assessed in the DG of the hippocampus in females and males under basal (grey bars) or chronic stress (black bars) conditions. A) Chronic stress reduced neuronal differentiation in females, but not males; there were no basal sex differences observed. B) Males and females did not differ in astroglial differentiation patterns under basal and chronic stress conditions. C) Representative image of the triple-immunfluorescent image (male non-stressed animal) with antibodies against BrdU (red), GFAP (violet) and NeuN (green). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.05$ *vs.* respective non-stressed group using a Mann Whitney *U* test (MWU). NS, non-stressed; S, stressed

Basal and chronic stress-induced sex differences on plasma CORT levels

To examine the effect of stress on CORT levels, basal blood samples were taken immediately prior to stress exposure on day 1, 3 and 5. The percentage CORT concentration as shown in Fig. 11 was calculated using the mean CORT concentration of male non-stressed animals on day 1 (397.6 ng/ml). Two-way repeated-measures ANOVA revealed a significant effect of sex ($F_{1,80}$=16.093; p=0.000), but not stress ($F_{1,80}$=2,151; p=0.147) or day ($F_{1,80}$=2.329; p=0.132) on plasma CORT levels. *Post-hoc* analyses showed that females express higher CORT levels on day 5 compared with males independent of stress condition (p<0.001; Fig. 11).

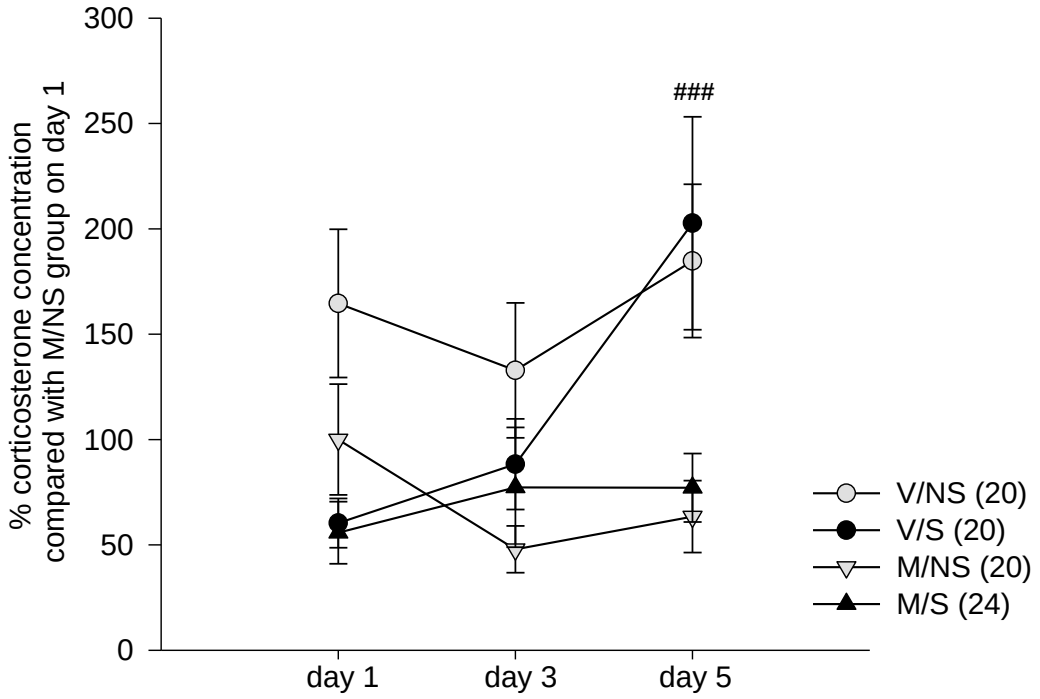

Figure 11: Effect of sex and chronic stress exposure on basal CORT levels

Plasma CORT levels on day 1, day 3 and day 5 were assessed immediately prior to chronic stress exposure. Females showed higher basal CORT levels across the time course compared to the respective male group. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. ###, p<0.001 *vs.* respective male group. NS, non-stressed; S, stressed

Basal and chronic stress-induced sex differences on spatial working memory

To examine naturally- occurring and sex-dependent differences in spatial working memory, males and females were tested in a single session on the Y-maze, one day after the end of chronic stress procedure. One- way ANOVA revealed no significant effects of sex ($F_{1,20}$=0.026; p=0.873) or stress ($F_{1,20}$= 0.259; p=0.616) on the percentage of alternations in the Y-maze (Fig. 12).

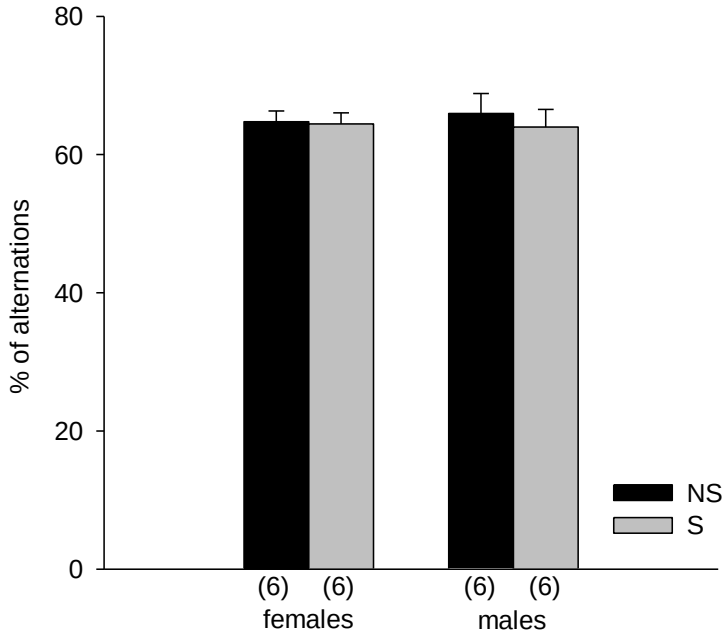

Figure 12: Effect of sex and chronic stress on spatial working memory in the Y-maze

Spatial memory task was performed one day after the end of chronic stress exposure in males and females. There was no basal or stress-induced difference in spatial working memory observed between males and females. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. NS, non-stressed; S, stressed

DISCUSSION

In the present study we demonstrate sex specific basal and chronic-stress induced differences in adult hippocampal neurogenesis. In males, chronic stress exposure reduced the hippocampal cell proliferation, which was associated with elevated stem cell quiescence, while the number of proliferating cells in females was unchanged. In contrast, cell survival under basal conditions was higher in females than in males, while chronic stress exposure affected this process specifically in females. Immature neuron production was higher in males than in females under basal and chronic stress conditions. While astroglial or neuronal differentiation patterns showed no basal sex-dependence, neuronal differentiation was reduced by stress in females only. Despite the basal and stress-induced differences in adult hippocampal neurogenesis, spatial working memory was shown to be independent of sex and chronic stress exposure, whereas basal CORT levels increased following stress exposure but only in females. Taken together, these results demonstrate both basal and stress-induced differences in adult hippocampal neurogenesis patterns that may underlie the differences in hippocampal morphology between sexes. To our knowledge these findings report the first direct comparison of sex-dependent and chronic stress-induced changes in hippocampal neurogenesis, which does not only assess cell proliferation.

Chronic stress impairs hippocampal neurogenesis in numerous species, in dependence on the intensity and frequency of stress exposure and timing of BrdU injection (Uno et al., 1989; Gould et al., 1997; Gould et al., 1998; Tanapat et al., 2001; Czeh et al., 2002; Pham et al., 2003; Torner et al., 2009) (for review see Abrous et al., 2005). Validating the efficiency of our

stress model, which has previously been used in neurogenesis studies (Magarinos and McEwen, 1995; Pham et al., 2003; Barha et al., 2011), we found a decreased body weight gain in males and females (data not shown), as well as a decreased absolute brain weight in males (data not shown). The observed decrease in absolute brain weight may relate to stress-induced atrophy, which has repeatedly been observed following stress exposure (Magarinos and McEwen, 1995; Conrad et al., 1996; Galea et al., 1997).

In accordance with previous work (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Pham et al., 2003; Heine et al., 2004), we revealed that chronic stress exposure attenuated cell proliferation in males, while exposure to chronic stress did not affect cell proliferation in females; despite basal levels not differing (Westenbroek et al., 2004). However, to date there are no studies available assessing basal and stress-induced differences in stem cell quiescence. We could show that under non-stressed conditions males and females expressed similar amounts of proliferating and quiescent stem cells. However, after 12 days of chronic RS there was a shift towards an increased quiescence in the hippocampal stem cell niche, but only in males, as revealed by an increased pool of label-retaining non-proliferating and undifferentiated cells that additionally expressed the stem cell maintenance marker SOX2. This shift in stem cells from a proliferating status to quiescence can partly explain the reduced proliferation rate seen after chronic stress in males. Thus, increased stem cell quiescence might be a mechanism to reconstitute the niche after depletion of precursors (Kazanis et al., 2010; Knobloch and Jessberger, 2011) and maintenance / preservation of the stem cell pool under pathophysiological situations (Kandasamy et al., 2010). Although, cellular quiescence seems to be actively maintained by distinct transcription programs (Coller et al., 2006), the exact regulatory mechanism of stem cell quiescence, particularly during chronic stress, are largely unknown. Therefore, future

studies are required, to assess molecules involved in the regulation of stem cell quiescence, such as the fibroblast growth factor (Palmer et al., 1995) under both basal and chronic stress conditions.

In addition and consistent with previous work, our study revealed, that there are not only robust stress-induced, but also baseline sex differences in adult hippocampal neurogenesis (Galea et al., 1997; Falconer and Galea, 2003; Westenbroek et al., 2004; Barha et al., 2011). Accordingly, we could show that males expressed a higher number of immature neurons in the DG despite a lower cell survival under basal conditions compared to females. However, whereas chronic stress led to a reduction in cell survival in females, it did not further reduce cell survival in males. These results are in accordance with previous work (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Pham et al., 2003; Heine et al., 2004; Westenbroek et al., 2004), and mirror the observed changes in neuronal differentiation patterns. Interestingly, chronic stress did not affect astroglial differentiation in either sex, but decreased neuronal differentiation; but only in females. These findings are in agreement with one previous study assessing cell differentiation following chronic stress in females (Barha et al., 2011) and illustrate that chronic stress particularly diminished the number of neurons; reflecting a diminished neuronal cell survival in females. With respect to the known increased susceptibility for women to suffer from stress-related illnesses like major depression (Kendler, 1998; Shors and Leuner, 2003; Becker and Grilo, 2007) and the fact that there seems to be a link between a reduced neurogenesis and depression (Czeh et al., 2001; Santarelli et al., 2003; Oomen et al., 2007; Snyder et al., 2011), this reduced neuronal fate in females may be of clinical relevance.

Stress-induced changes, which include in general an increase in glucocorticoid levels, are known to be crucial in regulating the effects of chronic stress on adult hippocampal

neurogenesis (Cameron and Gould, 1994; Lemaire et al., 1999; Tanapat et al., 2001; Hellsten et al., 2002). In the present study, we demonstrate that females exhibit higher basal CORT levels than males. Moreover, CORT levels further rose following chronic stress exposure specifically in females; as previously reported (Barha et al., 2011). In addition, during stress exposure females showed higher peak CORT levels than males (Romeo et al., 2004) and returned faster to baseline levels (McCormick and Mathews, 2007).

It is astonishing, that while females showed no effect of chronic stress on cell proliferation in the DG, the number of surviving newborn cells is almost halved despite an elevation in basal CORT levels, while males showed a contrary picture despite lower levels of CORT. However, cell proliferation might habituate to persistently elevated CORT levels (Czeh et al., 2001; Heine et al., 2004) as seen here in females. Moreover, the generation and removal of newborn cells are delicately balanced processes in the DG, which are counteracting each other (Biebl et al., 2000; Kuhn et al., 2001), which might also explain the discrepancy in cell proliferation and cell survival in males and females. Moreover, while CORT seems to be crucially involved in the regulation of hippocampal neurogenesis, it should be clearly kept in mind that there are other sex- as well as stress-related regulatory mechanisms that might affect neurogenesis. Two hormones that could be of relevance here are PRL and OXT, both of which attenuate hormonal and neuronal responses to stress (Pi and Grattan, 1999; Neumann et al., 2000b; Torner et al., 2004) and counteract stress-induced changes in neurogenesis (Torner et al., 2009; Leuner et al., 2010). Therefore, future studies will assess the involvement of these neuropeptides on the observed sex differences in hippocampal neurogenesis.

Another possible explanation for the present findings is related to the trisynaptic circuitry of the hippocampus. The decrease in granule cell number following stress in females reported

here in the DG, could be due to anterograde and retrograde projections that occur between both the CA3 and CA1 regions of the hippocampus (Witter, 1989, 1993). Arguing in favour of this theory is the fact that only a small percentage of precursor cells in the DG express CORT receptors (Cameron et al., 1993a). Thus, it is possible, that CORT may rather act indirect on neighbouring glial and neuronal cells that express appropriate receptors, which could then control the cell cycle of progenitor cells in the DG (Sousa and Almeida, 2002), *via* glutamate release in the DG (Stein-Behrens et al., 1994; Reagan and McEwen, 1997) or release of growth factors (Kuhn et al., 1997).

Learning and memory processes, especially spatial memory, have consistently been linked to adult hippocampal neurogenesis (Riedel et al., 1999; Shors et al., 2001; Leuner et al., 2006; Saxe et al., 2006; Kee et al., 2007; Snyder et al., 2009). Although, the cell types which particularly contribute to the effects in learning and memory are not fully elucidated, it seems that the production of immature neurons is functional relevant for this hippocampal function (Gould et al., 1999; Shors et al., 2001). Given the observed differences in the number of immature neurons between males and females and the fact that sex differences in memory tasks have already been described throughout species (de Frias et al., 2006; Shors, 2006a; Dalla et al., 2009; Dalla and Shors, 2009), a basal sex difference in spatial working memory would have been expected. However, there was neither an effect of sex, nor of chronic stress exposure on spatial working memory. Admittedly, the specific functions contributed by adult-born neurons remain controversial and there is growing evidence that they might be rather involved in the acquisition of emotional relevant information (Hernandez-Rabaza et al., 2009) and the regulation of anxiety (Revest et al., 2009; Dagyte et al., 2011). Therefore, future studies will assess anxiety-related behaviour to reveal a

potential link between differences in the number of immature neurons and this type of affective behaviour in males and females.

CONCLUSIONS

The results of the present study indicate that exposure to chronic RS alters the neurogenic and CORT status in a sex-dependent manner. While chronic stress exposure only led to short-term alterations in neurogenesis in males, long-term consequences were observed in females, particularly in neuronal differentiation. Moreover, the present results show that immature neuron production does not necessarily predict spatial working memory. Further research is required to elucidate the counteracting mechanisms that underlie the different stages of hippocampal neurogenesis in males and females under basal and under conditions of stress.

To our knowledge this is the first direct comparison of gender-dependent and chronic stress-induced changes in adult hippocampal neurogenesis, which does not only assess cell proliferation and survival, but also includes the analysis of differentiation stages and stem cell quiescence in the DG.

Chapter 4

Reversal of lactation-induced reduction in hippocampal neurogenesis by chronic stress

Author's contribution:

Hillerer: Study design, performing of experiments, analyzing data, writing the manuscript

Neumann: Study design

Couillard-Despres: Establishment of staining, contribution to manuscript writing

Aigner: Study design, contribution to manuscript writing

Slattery: Study design, performing experiments, contribution to manuscript writing

[adapted from: Hillerer K.M., Neumann I.D., Couillard-Despres S., Aigner L., Slattery D.A. Reversal of lactation-induced reduction in hippocampal neurogenesis by chronic stress. Neuroscience, in preparation]

ABSTRACT

Several factors like acute and chronic stress, and perhaps unexpectedly, lactation have been shown to decrease hippocampal neurogenesis. Lactation has been shown to be a time of reduced stress responsivity, but the consequence of stress during lactation on hippocampal neurogenesis remains unknown. Therefore, the aim of the present study was to assess the effect of chronic stress during lactation (2 h RS from LD2 to LD13) on cell proliferation and survival, neuronal and astroglial differentiation and stem cells quiescence. In addition, CORT levels were assessed. Beside the replication of the known lactation-associated decrease in cell proliferation and survival, we revealed that chronic stress reversed this decrease in cell proliferation, despite an increase in quiescent stem cells and an unchanged cell survival. Whereas chronic stress did not alter immature neuron production, it specifically diminished the number of mature neurons in lactating stressed dams, while the number of astroglial cells was unaffected. Basal CORT levels were shown to be increased in stressed dams. To our knowledge, this is the first study that assesses the effect of chronic stress during lactation on hippocampal neurogenesis and indicates that chronic stress during lactation interferes with important peripartum adaptations at the level of the hippocampus.

INTRODUCTION

Throughout all mammalian species, motherhood is characterised by numerous neuroendocrine and behavioural adaptations. Among these, the most obvious are lactogenesis and milk ejection and maternal behaviour, including maternal care and aggression, (Walker et al., 1995; Neumann, 2001; Russell et al., 2001; Hillerer et al., 2011a). However, in addition to changes directly associated with reproductive functioning, a host of important behavioural and physiological alterations occur (Walker et al., 1995; Carter et al., 2001; Neumann, 2001, 2003), with decreased anxiety / increased calmness (Altemus et al., 1995; Carter et al., 2001; Heinrichs et al., 2001) and increased basal cortisol / CORT levels among the most prominent. The increased level of circulating basal glucocorticoids are related to the presence of nursing pups (Stern et al., 1973; Walker et al., 1992) and is also directly correlated with the concomitant decrease in hippocampal neurogenesis observed in the peripartum period (Leuner et al., 2007).

In addition to the peripartum-associated hypercorticism / hypercortisolism, there are numerous alterations which act in concert to decrease the response of HPA axis to external stressors (Altemus et al., 1995; Heinrichs et al., 2001; Brunton et al., 2008). This raises the intriguing possibility that stress during the peripartum period affects neurogenesis in a different fashion to that observed in male and female (virgin) rodents. Thus, it has repeatedly been demonstrated that exposure to a variety of chronic stressors decreases cell proliferation and survival in males, and cell survival in females (Gould et al., 1997; Gould et al., 1999; Lucassen et al., 2001; Pham et al., 2003). Such stress-related alterations in

neurogenesis have been posited as playing a crucial role in the aetiology of anxiety and depression (Fuchs, 2007). Moreover, there is a growing body of evidence suggesting that antidepressants mediate their effect, at least in part, by increasing hippocampal neurogenesis (Hanson et al., 2011; Snyder et al., 2011). Therefore, as the peripartum period is known to be a time of increased susceptibility for mood and anxiety disorders (Robertson et al., 2004; Beck, 2006; Lonstein, 2007; Bridges, 2008) and stress during pregnancy and lactation is one of the most prominent risk factors to develop postpartum mood disorders (Robertson et al., 2004), it may be hypothesized that alterations in hippocampal neurogenesis may underlie the observed disturbances in postpartum mood. However, previous studies have not explored an impact of stress during lactation on postpartum hippocampal neurogenesis.

Therefore, the aim of our study was to determine the effects of chronic RS during the lactation period on different aspects of hippocampal neurogenesis. To more specifically examine chronic stress effects on neurogenesis in the DG of the hippocampus, we did not only evaluate the number of proliferating and surviving cells, but moreover, assessed neuronal and astroglial differentiation patterns, as well as stem cell quiescence. In addition basal CORT levels were assessed, to establish whether chronic stress during lactation prevents the hypercorticism typically seen during lactation and whether there is a causal relationship to changes in cell proliferation and cell fate.

MATERIALS AND METHODS

Animals

Female (200-250g) Wistar rats, 9-10 weeks old (Charles River, Sulzfeld, Germany) were housed in groups of four in standard polycarbonate rat cages and allowed to habituate for at least seven days. All rats were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00h, 22°C ± 1°C, 60 55 ± 5% humidity) and had free access to water and standard rat diet. All experimental procedures were performed between 08:00 – 12:00 A.M., approved by the Committee on Animal Health and Care of the local government of the Oberpfalz, and complied with international guidelines on ethical use of animals.

Mating procedure and confirmation of pregnancy

After habituation, all female rats were mated (two to three females/male); and pregnancy was verified by vaginal smears (designated PD0). Nonpregnant rats were assumed to be nulliparous (equates to virgins in the following text). All animals were returned to group cages with 4 females housed together (cage size 55 x 35 x 20 cm) until PD16, when they were single-housed. On the day of birth, i.e. LD1, the number of pups in the litter and the average birth weight were determined, and then all litters were culled to 8 pups to ensure comparable conditions across all dams.

Chronic stress procedure

Animals of stressed group were subjected to 2 h RS for 12 consecutive days (LD2-LD13, or equivalent in virgins) between 10:00 - 12:00 A.M. RS has been repeatedly shown to be an effective stressor in female rats (Darnaudery et al., 2004; Hillerer et al., 2011a; Hillerer et al., 2011b) and to affect proliferation and survival rate of new hippocampal cells (Pham et al., 2003; Luo et al., 2005; Rosenbrock et al., 2005). Each rat was placed in a plexiglass column with ventilation holes (12 cm diameter). Non-stressed controls were single-housed and left undisturbed in their home cages in the same room. The body weight of each animal was recorded daily from the beginning of the stress procedure until the day of sacrifice.

Virgins

In order to determine the effects of the reproductive status and chronic stress on hippocampal neurogenesis in lactating females, the present study was carried out in parallel with that of chapter 3. Therefore, we reused the data on cell proliferation, cell survival, differentiation patterns and stem cell quiescence from the virgin groups to determine whether the observed effects were specific to the peripartum period or to females in general.

Experiments 1 and 2: Assessment of hippocampal cell proliferation, cell survival, cell fate and stem cell quiescence

BrdU labelling

To examine the proliferation of precursor cells, rats were injected with 5-Bromo-2'-desoxyuridine (BrdU; 50mg/kg, i.p.) once, on the last day of stress, immediately after removal from the restraint tubes, i.e at 12:00 A.M.. 24 h after BrdU injection animals were deeply anesthetized with ketamine/ xylazine (90-120 mg/kg ketamine and 6-8mg /kg xylazin diluted in 0.9% NaCl), intra-cardiacally perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. 24 h have been reported to be sufficient for newborn cells to complete one cell cycle (Takahashi et al., 1992).

To trace the survival and fate of recently born cells, rats received daily injections of BrdU (50mg/kg, i.p.) during the first four days of stress exposure (LD2-LD5, or equivalent in virgins) as described above. 16 days after the last BrdU injection (LD21, or equivalent day in virgins) rats were anesthetized and perfused as described above. BrdU (Sigma/Aldrich) was freshly prepared in 0.9% NaCl solution to a dilution of 20mg/ml on each injection day.

(For schematic representation of the temporal design of the study see *Figure 13* of the result section)

Histological procedures

Immediately after the perfusion brains were post-fixed in 4% paraformaldehyde at 4°C overnight, before transfer to 30% sucrose, 0.1 M sodium phosphate solution (pH 7.4) in sterile water for at least one week. Sagital brain sections (40µm) were prepared using a

sliding microtome on dry ice and subsequently stored at 4°C in a cryoprotection solution (glycerol, ethylene glycol and 0.1 M phosphate buffer, pH 7.4, at a ratio of 1:1:2 by volume; (Kandasamy et al., 2010)). Immunostaining of BrdU-labelled cells was performed on free-floating sections using the DAB peroxiodase method. Briefly, brain sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min. For DNA denaturation, sections were incubated for 2 h in 50% formaldehyde/2 X saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, rinsed for 5 min in 2 X SSC, incubated in 2M HCl for 30 min at 37°C and washed for 10 min in 0.1M boric acid, pH 8.5. Thereafter, sections were incubated in FSGB for 1 h, followed by incubation with the primary rat α- BrdU antibody (1:500, Oxford Biotechnology, Oxford, UK) in FSGB overnight at 4°C. The next day, the sections were incubated with biotinylated secondary donkey α-rat antibody (1:500,Molecular Probes), followed by the avidin-biotin-peroxidase complex reaction (1 h; Vectastain elite ABC kit; Vector Laboratories, Burlingame, CA (Kandasamy et al., 2010)). Thereafter, the signal was visualized using DAB (25mg/ml in water with 0.01% H_2O_2, 0.04 $NiCl_2$). Stained slides were mounted on microscopic slides, washed with NeoClear (Merck) and cover-slipped with NeoMount (Merck). Immunostaining of DCX-labelled cells was performed on free-floating sections as described above (antibodies used: 1° AB goat α DCX , 1:250 in FSGB (Santa Cruz Biotechology; Santa Cruz, CA); 2° AB biotinylated donkey α goat (Molecular Probes); (Kandasamy et al., 2010)). Triple-immunfluorescence for BrdU/ GFAP/ NeuN was performed using a standardised protocol (Kandasamy et al., 2010). Briefly, free floating brain sections were incubated in 50% formaldehyde/2 X saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) at 65°c for 1 h, rinsed for 5 min in 2 X SSC, incubated in 2M HCl for 30 min at 37°C and washed for 10 min in 0.1M boric acid, pH 8.5. Thereafter, sections were washed four times in TBS for 5 min, before they were incubated

with FSGB for 30 min, followed by incubation with the antibody mix (BrdU α rat, 1:500,Oxford Biotechnology, Oxford, UK; GFAP α guinea pig, 1:500, Progen; NeuN α mouse, 1:500, Chemicon) in FSGB for 48h at 4°C. 48h later, the sections were incubated overnight with the secondary antibody mix (donkey α rat conjugated with rhodamine red, 1.500; donkey α guinea pig conjugated with IgG Cy5, 1:500; donkey α mouse conjugated with Alexa Fluor 488, 1.500) in FSGB, shaking in the dark. Stained slides were mounted on microscopic slides and cover-slipped with Prolong- Antifade (Molecular Probes). Triple-immunfluorescence for BrdU/PCNA/ SOX2 was performed as described above (used antibodies: 1°AB: BrdU α rat, 1:500, Oxford Biotechnology, Oxford, UK; PCNA α mouse, 1.500, Santa Cruz Biotechnology, Santa Cruz, CA; SOX2 α goat , 1:500, Santa Cruz Biotechnology, Santa Cruz, CA; 2°AB: donkey α rat conjugated with rhodamine red, 1.500; donkey α goat conjugated with Alexa Fluor 660, 1:500; donkey α mouse conjugated with Alexa Fluor 488, 1:500; (Kandasamy et al., 2010)).

Stereology

To determine the number of BrdU-positive cells, every sixth section (240µm interval) of the right hemisphere was examined for BrdU- positive cells throughout the rostral caudal extent of the granule cell layer and the adjacent SGZ. Cells were counted regardless of shape or size under 10X magnification. We used a semi-automatic stereology system (Stereoinvestigator, MicroBrightField) and a 5X objective to trace a defined area of the DG / SGZ. The defined area was used to calculate the number of BrdU-positive cells per $100m^2$ of the DG.

Confocal analysis

All morphological analyses were performed by an experimenter blind to the group. To determine the frequency of neuronal differentiation of newborn cells, a series was examined using a grid confocal laser microscope (Olympus XI81) using every sixth section (240µm interval). Z-stacks were built using the Volocity Software (Perkin Elmer). 50 BrdU-positive labelled cells per animal were analysed for neuronal differentiation. BrdU-positive cells were counted as solely BrdU-positive (newborn cells), BrdU/ NeuN (newborn neurons) double-positive cells and BrdU/GFAP (newborn astrocytes) double-positive cells. The same procedure was performed to determine the frequency of quiescent stem cells. BrdU-positive cells were counted as solely BrdU-positive (newborn cells), BrdU-positive/PCNA-negative/SOX2-positive (quiescent stem cells) and BrdU-positive/ PCNA-positive/ SOX2-positive (proliferating stem cells)

Experiment 3: Effect of stress exposure on plasma CORT levels

As CORT levels have been shown to be an important regulator of neurogenesis (Magarinos and McEwen, 1995; Galea et al., 2008; Barha et al., 2011), we measured CORT levels under basal and stress conditions. Therefore, a jugular vein surgery was performed on PD19 as previously described (Neumann et al., 1998b; Bosch et al., 2007b; Hillerer et al., 2011b). Briefly, the jugular vein was exposed by blunt dissection, then a catheter consisting of silicon tubing (Dow Corning Corp., Midland MI, USA) and PE-50 polyethylene tubing was inserted approximately 3 cm into the vessel through an incision in a cardiac direction and exteriorized at the neck of the animal. The catheter was filled with sterile saline containing gentamicin (30,000IU/ml; Centravet, Bad Bentheim, Germany). Five days after surgery, on the first day

of chronic stress procedure (LD2), LD4 and LD6 at 07:30, the catheter was attached to an extension tube connected to a 1-ml plastic syringe filled with sterilized heparinised 0.9% saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany). Each rat was then left undisturbed for 2 h. Two basal samples (basal sample one: 0.6ml and basal sample two: 0.2ml) were taken 30 min apart and were used to calculate the mean basal concentrations for CORT. After the sampling of basal sample 2 rats were placed into the restraint tubes. After 2 h rats were removed from the restraint tubes and put back in their home-cage. All blood samples were immediately replaced with the same volume of intravenous sterile 0.9% saline. All blood samples were collected on ice in EDTA-tubes containing aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) and analysed for CORT using a commercially available ELISA kit.

Statistical analysis

All numerical data are expressed as the mean ± SEM and statistically analysed using an unpaired Student's t-test, a Mann Whitney *U* test, or a one- or two- way analysis of variance (ANOVA) with or without repeated measures, as appropriate. Any statistical differences, which were set at $p< 0.05$, were further analysed using a Fisher's *post-hoc* test. Statistical analyses were performed using SPSS for Windows (version 16; SPSS Inc, Chicago, IL, USA).

RESULTS

Effect of reproductive status and chronic stress on cell proliferation

To assess the effects of chronic stress during lactation on cell proliferation, animals were given a single BrdU injection and perfused 24 h later (see *Figure 13A*). Two-way ANOVA revealed that cell proliferation was altered by the reproductive status ($F_{1,23}$=26.449; p<0.001) and by chronic stress exposure ($F_{1,23}$=7.395; p=0.012). *Post-hoc* analyses revealed that lactating animals showed a reduction in cell proliferation both under basal and chronic stress conditions (p<0.05; Fig. 14A). Additionally, lactating stressed animals showed an increased proliferation rate compared to non-stressed dams (p<0.01; Fig. 14A).

Effect of reproductive status and chronic stress on stem cell quiescence

We next examined the effect of chronic stress on the number of quiescent and proliferating stem cells. Therefore, animals received BrdU injections on the first 4 days of stress (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 13B*). The number of quiescent stem cells (BrdU positive, SOX2 positive, PCNA negative) was independent of the reproductive status ($F_{1,21}$=2.387; p=0.137) or chronic stress ($F_{1,21}$=3.945; p=0.060). However, separate analysis confirmed an increase in the number of quiescent stem cells in the DG of stressed lactating dams compared with the respective non-stressed group (Mann Whitney *U*; p=0.003; Fig. 14B). Moreover, a trend towards a decreased quiescence was observed in lactating non-stressed animals compared with the respective virgin group (t_{10}=-2,023; p=0.071; Fig. 14B). Analysis of the percentage of proliferating stem cells (BrdU positive, SOX2 positive, PCNA positive; Fig. 14C) revealed no significant effect of

reproductive status ($F_{1,21}$=0.280; p=0.602) or chronic stress ($F_{1,21}$=4.073; p=0.057). However, a separate Mann Whitney *U* analysis showed a decrease in the percentage of proliferating stem cells in stressed lactating animals compared with non-stressed dams (p=0.022; Fig. 14C).

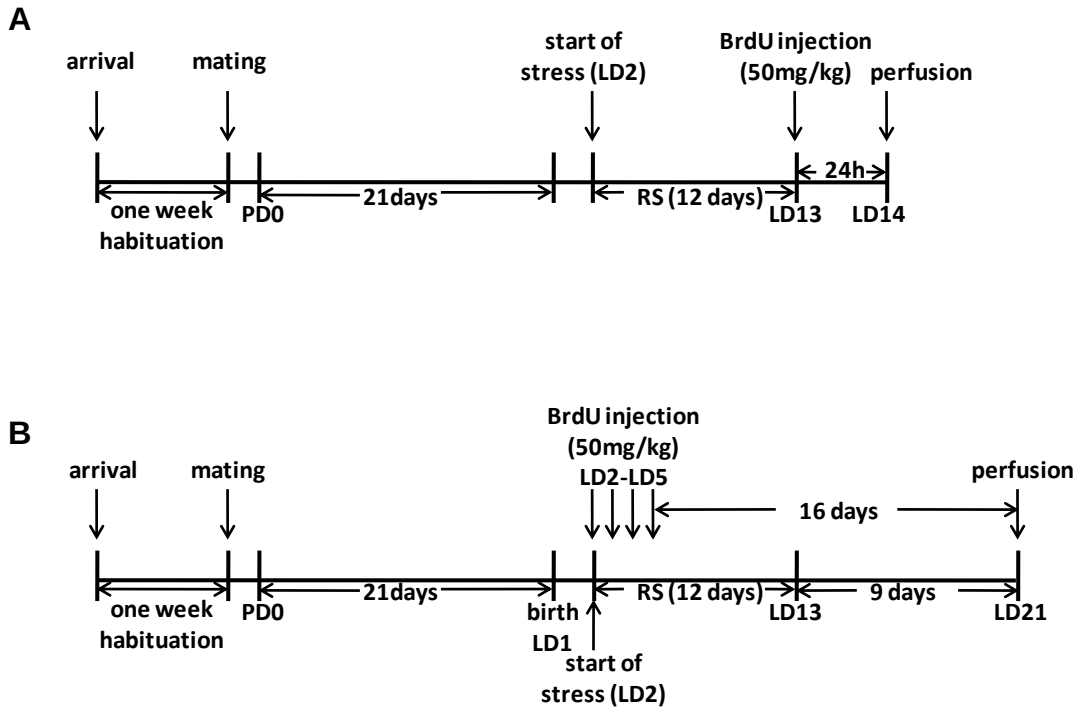

Figure 13: Schematic representation of the temporal designs used in the studies

Temporal designs used to assess A) cell proliferation, immature neuron production (DCX positive cells) and B) cell survival, neuronal/ astroglial differentiation and quiescent/ proliferating stem cells. After one week of habituation, all females were mated. Animals were exposed to 2 h RS from LD2 to LD13 (or equivalent in virgins). They received either A) a single BrdU injection (50mg/kg; i.p.) on the last day of stress (LD13 or equivalent in virgins) or B) four BrdU injections (50mg/kg; i.p.) on 4 consecutive days (LD2-LD5 or equivalent in virgins). Animals were intra-cardiacally perfused either A) 24 h after (LD14 or equivalent in virgins) or B) 16 days after (LD21 or equivalent in virgins) the last BrdU injection.

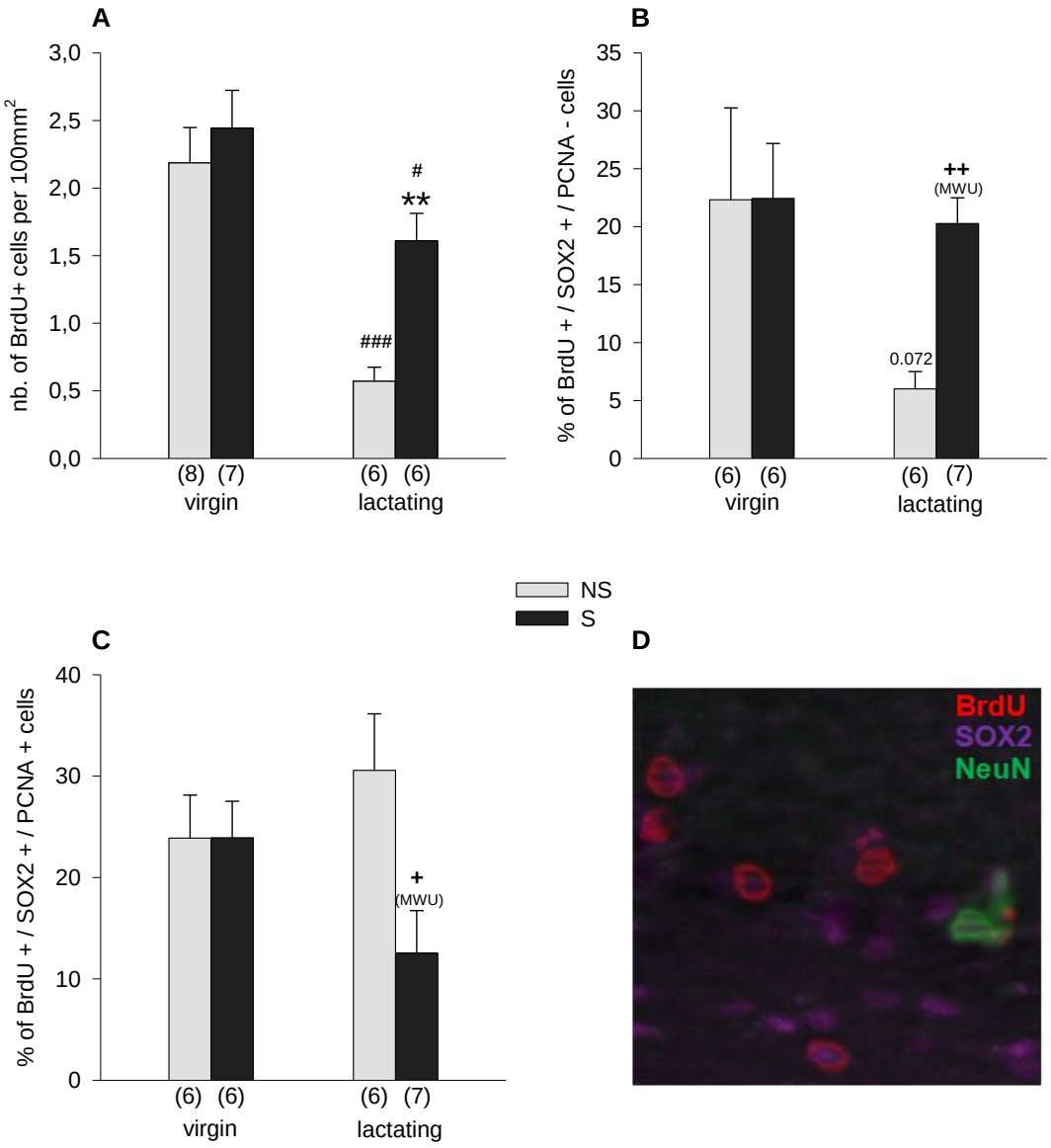

Figure 14: Effect of reproductive status and chronic stress on cell proliferation and stem cell quiescence

The total number of proliferating cells (A), the percentage of quiescent stem cells (B) and the percentage of proliferating stem cells (C) was assessed in the DG of the hippocampus in virgin and lactating animals under basal (grey bars) or chronic stress (black bars) conditions. A) Lactating animals showed a lower number of proliferating cells, both under basal and chronic stress conditions; stress during lactation led to increase in cell proliferation when compared with the respective non-stressed group. B) Analysis of the number of BrdU positive, SOX2 positive and PCNA negative cells, revealed an increase in the percentage of quiescent stem cells after chronic stress in lactating dams; separate analyses showed a trend towards a decrease in the percentage of quiescent cells in lactating non-stressed *vs*. respective virgin group. C) Chronic stress reduced the number of proliferating stem cells (BrdU positive, SOX2 positive, PCNA positive) in lactating, but not virgin animals; no reproductive status effect was observed. D) Representative image of the triple-immunfluorescent image (lactating non-stressed animal) with antibodies against BrdU (red), SOX2 (violet) and PCNA (green). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. **, p<0.01 *vs.* respective virgin group; ###, p<0.001 *vs.* respective non-stressed group; +, p<0.05 *vs.* respective non-stressed group using a Mann Whitney *U* test (MWU); ++, p<0.01 *vs.* respective non-stressed group using a Mann Whitney *U* test (MWU); 0.072 *vs.* respective virgin group using an unpaired Student's t-test. NS, non-stressed; S, stressed

Effect of reproductive status and chronic stress on immature neuron production and cell survival

To examine the effect of reproductive status and chronic stress exposure on the number of immature neurons (DCX positive cells), animals were given a single BrdU injection and perfused 24 h later (see *Figure 13A*). There was no significant effect of reproductive status ($F_{1,24}$=0.457; p=0.505) or stress ($F_{1,24}$=0.129; p=0.723) observed on the number of immature neurons in the DG (Fig. 15A). A significant interaction between reproductive status and stress ($F_{1,21}$=4.469; p=0.044) was shown with respect to the number of surviving cells. Separate analyses revealed a reduction in cell survival in non-stressed lactating compared to virgin animals ($p<0.01$; Fig. 15B) and a reduced survival in virgin females after chronic stress exposure ($p>0.05$; Fig. 15B).

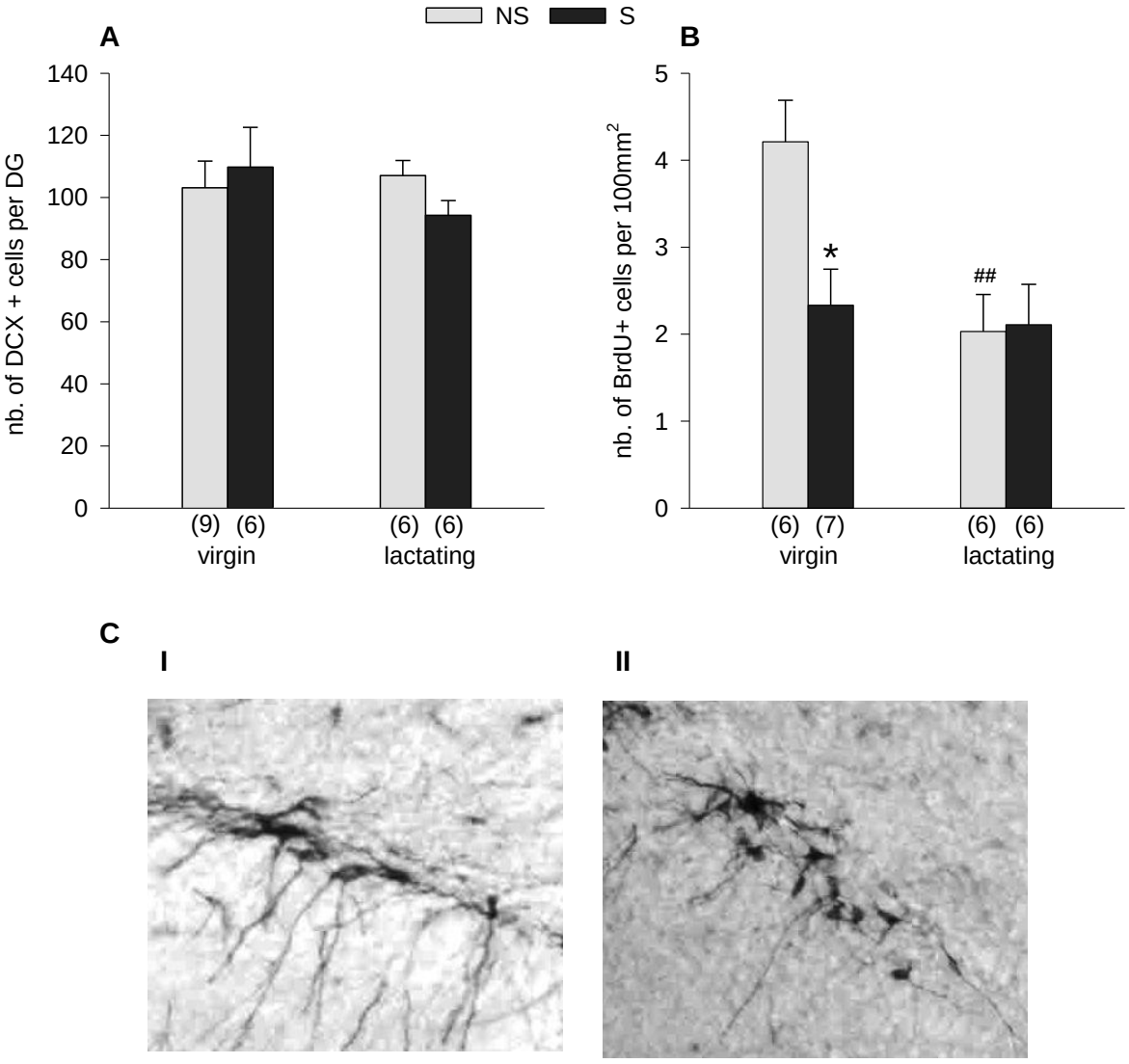

Figure 15: Effect of reproductive status and chronic stress on immature neuron production and survival of cells

The number of immature neurons (DCX+ cells) (A) and the number of surviving cells (B) was assessed in the DG of the hippocampus in virgin and lactating animals under basal (grey bars) or chronic stress (black bars) conditions. A) There was no effect of reproductive status or stress on immature neuron production; B) Lactating animals showed a reduction in cell survival under non-stress conditions; stress led to a reduction in cell survival in virgins, but not lactating animals; C) Representative image of a DAB-immunostaining (lactating non-stressed animal (I) and lactating stressed animal (II)) against DCX. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.01$ *vs.* respective non-stressed group; ##, $p<0.01$ *vs.* respective virgin group. NS, non-stressed; S, stressed

Effect of reproductive status and chronic stress on neuronal and astroglial differentiation

To evaluate if virgin and lactating animals vary in their astroglial and neuronal differentiation patterns under basal and chronic stress conditions, animals received BrdU injections on the first 4 days of stress (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 13B*). There was no effect of reproductive status on astroglial ($F_{1,23}$= 2.321; p=0.142) or neuronal ($F_{1,23}$=0.001; p=0.972) differentiation. Separate analyses revealed an effect of stress on neuronal ($F_{1,23}$=11.535; p=0.003; Fig. 16A), but not astroglial ($F_{1,23}$=2.321; p=0.142; Fig. 16B) differentiation with *post-hoc* analysis confirming a reduced neuronal differentiation in virgins and dams that were exposed to stress (p<0.05; Fig. 16A).

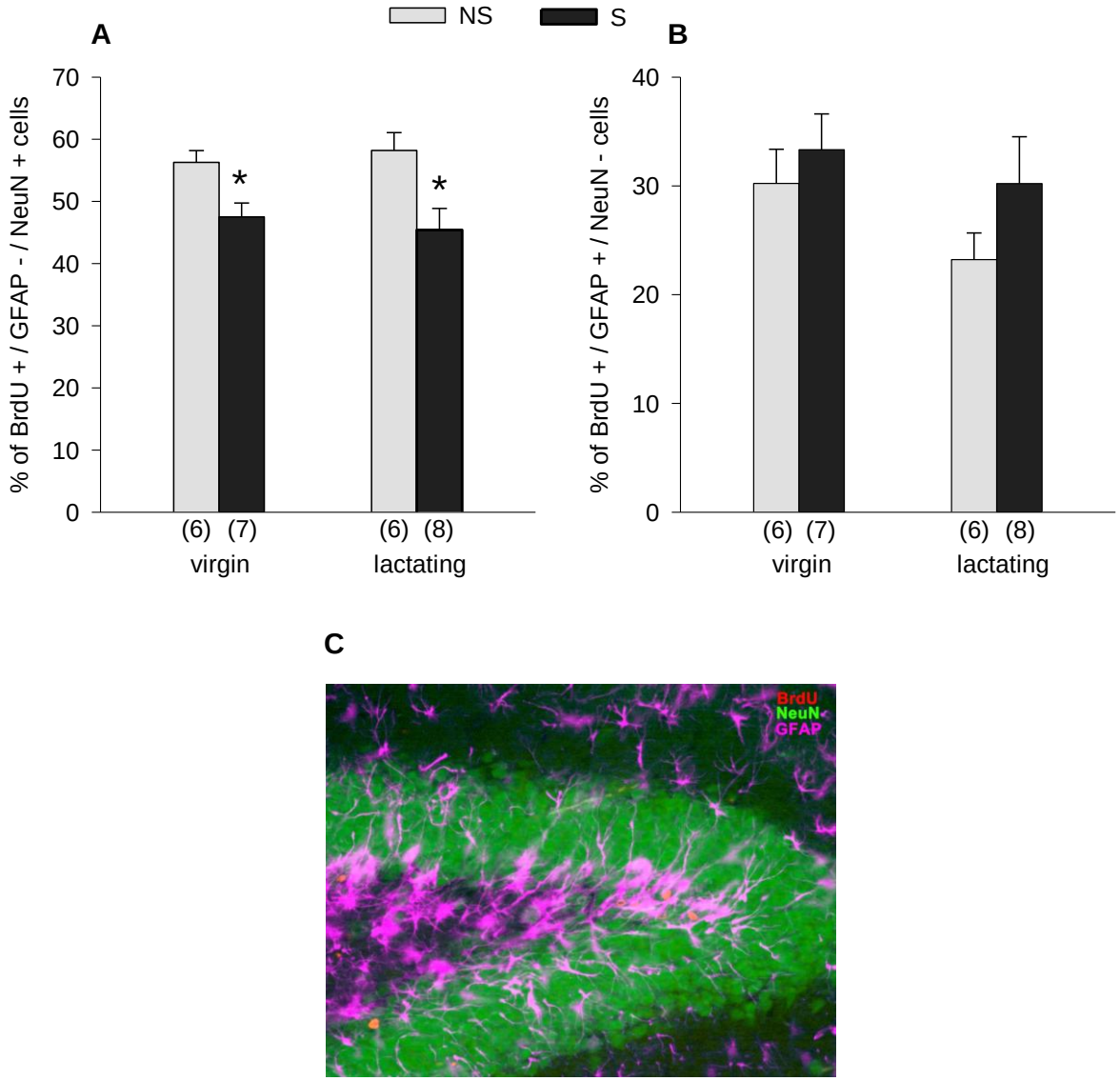

Figure 16: Effect of reproductive status and chronic stress on differentiation patterns

The percentage of cells differentiating into neurons (BrdU positive, GFAP negative and NeuN positive cells) (A) and astroglial cells (BrdU positive, GFAP positive, NeuN negative cells) (B) was assessed in the DG of the hippocampus in virgin and lactating animals under basal (grey bars) or chronic stress (black bars) conditions. A) Chronic stress reduced neuronal differentiation in virgin and lactating females, no basal differences were observed between virgin and lactating animals. B) There was no effect of the reproductive status or chronic stress on astroglial differentiation patterns. C) Representative image of the triple-immunfluorescent image (lactating non-stressed animal) with antibodies against BrdU (red), GFAP (violet) and NeuN (green). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.05$ *vs.* respective non-stressed group. NS, non-stressed; S, stressed

Effect of chronic stress on plasma CORT levels

To assess the effect of chronic stress during lactation on CORT levels, basal blood samples were taken immediately prior to stress exposure on LD2, LD4 and LD6. Two way repeated-measures ANOVA revealed a significant interaction effect of day and stress ($F_{1,28}$=9.042; p=0.006). *Post-hoc* analysis showed that stressed dams had higher basal CORT levels on LD6 compared with non-stressed dams (p<0.01; Fig. 17).

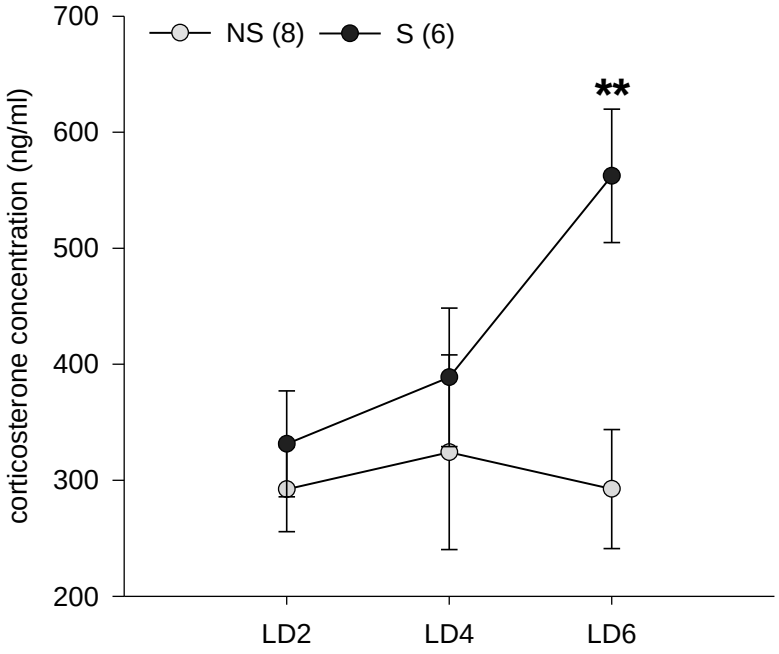

Figure 17: Effect of chronic stress on plasma CORT levels

Plasma CORT levels on LD2, LD4 and LD6 were assessed immediately prior to chronic stress exposure. Stressed dams showed higher basal CORT levels on LD6 when compared with the non-stressed dams. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. **, p<0.01 *vs.* respective non-stressed group. NS, non-stressed; S, stressed

DISCUSSION

This study provides the first evidence that different distinct stages of adult hippocampal neurogenesis are affected by chronic stress during the postpartum period. As previously reported, lactating dams showed a decrease in cell proliferation in the DG of the hippocampus compared with virgins (Darnaudery et al., 2007; Leuner et al., 2007), which was reversed by chronic stress exposure. Chronically stressed dams displayed a lower percentage of surviving cells, as well as a reduced neuronal differentiation, whereas immature neuron production and astroglial differentiation were unaffected by chronic lactation stress. Interestingly, while quiescence of stem cells tended towards a downregulation in lactating animals compared with virgins, chronic stress prevented this postpartum neuronal adaptation. Moreover, the results of the present study reveal that basal CORT levels are only partly involved in the regulation of adult hippocampal neurogenesis under stress conditions during lactation, which further suggests that fluctuations in other hormones that occur classically during the peripartum period may play a major role. Collectively, these findings highlight that chronic stress during lactation has detrimental effects on postpartum-associated adaptations at the level of the hippocampus.

Chronic stress during lactation prevents the lactation-associated decrease in hippocampal cell proliferation and leads to a decrease in cell survival

Hippocampal morphology undergoes a postpartum reorganization (Galea et al., 2000; Oatridge et al., 2002), which includes dendritic pruning of the CA3 and CA1 pyramidal neurons of the hippocampus at the time of weaning, as well as an increase in spine density

during pregnancy and lactation (Kinsley et al., 2006). Moreover, and consistent with our data, a decrease in hippocampal cell proliferation and cell survival (Darnaudery et al., 2007; Leuner et al., 2007; Pawluski and Galea, 2007) has been shown to be a phenomenon related to the postpartum period and is associated with the hypercorticism during that time (Leuner et al., 2007). Interestingly, we could show that the lactation-associated decrease in cell proliferation was prevented by chronic stress exposure, but actually led to a decrease in cell survival. Stressed dams showed an activation of the HPA axis across the tested lactation period with higher basal CORT levels observed on LD6 compared with non-stressed dams. These results may initially seem paradoxical, as chronic psychosocial and physical stress are commonly thought to impair adult hippocampal neurogenesis (Gould et al., 1997; Czeh et al., 2002; Pham et al., 2003; Heine et al., 2005), mainly due to an effect of increased basal CORT levels (Gould et al., 1992; Cameron and Gould, 1994; Tanapat et al., 2001). However, the latter studies have been performed in males and studies in females show that chronic stress reduces cell survival (Kuipers et al., 2006) without a change in the number of proliferating cells (Falconer and Galea, 2003; Westenbroek et al., 2004). Thus, it might be that females react different to chronic stress situation and elevated CORT levels. Additionally, it has been shown that the proliferation rate in the DG may habituate to chronic stress and this may display a reduced sensitivity to HPA axis hormones (Czeh et al., 2002). Despite the literature supporting a link between a reduction in adult hippocampal neurogenesis and high basal CORT levels during chronic stress and lactation, only a small percentage of precursor cells express CORT receptors (Cameron et al., 1993a). Hence, there are other regulatory mechanisms that could play an important role under chronic stress conditions. Further, the generation and removal of cells is delicately balanced and incorporation of newborn cells above a critical set-point might also be maladaptive as

observed following epileptic seizures (Varodayan et al., 2009). This may also apply to the special situation of lactation, as the normal decreased cell proliferation may be involved in the reduced stress responsiveness observed at this time (Neumann, 2001; Lonstein, 2007). In addition, closer inspection of hippocampal cell proliferation and cell survival in both lactation groups revealed that a higher percentage of cells died in stressed dams over a distinct time course than in non-stressed dams. This suggests that the lactation period, under normal circumstances, is associated with a greater efficiency in neurogenesis processes, as fewer proliferating cells are required to result in the same net cell survival as occurs in virgins / males, which is in accordance with previous studies (Cameron et al., 1993b; Tanapat et al., 2001). In contrast, increased cell death occurred in virgins despite no changes in cell proliferation. Thus, increased efficiency of neurogenesis during the lactation period might be an evolutionary adaptation, as the generation of new cells is a highly metabolic resources taking process and the dam requires high metabolic resources to successfully raise her offspring. Furthermore, the observed results indicate that either apoptotic processes of progenitor cells and / or cell cycle length differs between the two lactating groups. The discrepancy between cell proliferation and cell survival in the stressed dams could indicate an increased apoptosis of newly generated cells and, thereby, be in accordance with other literature, suggesting a compensatory increase in cell proliferation as a result of an increased cell death of older cells after chronic stress exposure (Gould et al., 1991a; Lucassen et al., 2001; Abrous et al., 2005).

Another possible explanation of the discrepancies between stressed and non-stressed dams is an alteration in cell cycle length. Interestingly, increased levels of cell proliferation in epilepsy are not only associated with an increase in the apoptotic rate, but moreover with a shortened cell cycle length (Varodayan et al., 2009). Therefore, it can be hypothesized that

the turnover of the DG is accelerated by an increased division rate in stressed dams. In this regard, oestrogen could play an important role, which decreases in lactation from high levels in pregnancy and stimulates a population of DG granule cells to divide faster, by regulation of the G_1/S-phase transmission of the cell cycle (Geum et al., 1997; Hong et al., 1998). Therefore, chronic stress may maintain high oestrogen levels in the dams, which, at least in part, would explain the observed changes in hippocampal cell proliferation and cell survival. Future studies will therefore assess apoptotic rates and cell cycle length by use of specific markers (i.e. Ki-67), as well as oestrogen levels, to get a better insight in factors regulating cell proliferation and cell survival under chronic stress conditions during lactation.

Chronic stress during lactation does not affect immature neuron production, but abolishes the lactation-associated decrease in stem cell quiescence and leads to an alteration in astroglial and neuronal differentiation patterns

In addition to proliferation and survival, selection, differentiation and integration of adult generated cells into the circuitry are important components of neurogenesis (Lledo et al., 2006). Disruption of these processes by environmental changes or chronic stress exposure may cause a lack of cell populations with specific properties in hippocampal function (Abrous et al., 2005). Although immature neuron production was found to be decreased in a previous lactation study (Leuner et al., 2007) we did not find an effect of lactation on the number of DCX+ cells. However, differences in the outcome of results might be explained by the use of a different neuronal lineage marker in the study by Leuner et al. (TuJ1). In accordance with the results in male rats, where three weeks of RS did not alter immature neuron production (Pham et al., 2003), and virgin rats in the present study, chronic stress during lactation had

also no effect on neuronal progenitor cells. These results might initially not fit to the observed changes seen in cell proliferation and cell survival, but on closer inspection, there is a trend towards a decrease in immature neuron production, that might facilitate the interpretation of received results. With respect to the hypothesized increase in apoptosis, it seems feasible that the unchanged immature neuron production in stressed dams mirrors the shift from the observed increased proliferation towards the decreased survival, as occurred in virgins. This becomes even more evident when considering the neuronal and astroglial differentiation patterns. Whereas neuronal differentiation was unchanged during the normal lactation period, chronic stress led to a reduction in mature neurons with a concurrent trend towards an increase in mature astroglial cells, although this phenotypic differentiation was slightly decreased as an effect of undisturbed lactation. These results reveal that cells that are engaged in apoptotic processes in the postpartum stress group are solely neurons. The addition of new neurons that reach maturity is thought to be of importance in relation to motherhood, especially maternal bonding and care (Gandelmann et al., 1971; Santarelli et al., 2003; Leuner and Shors, 2006) and *vice versa* (Furuta and Bridges, 2009). Therefore, alterations in the number of neurons within the hippocampus might be directly, and indirectly *via* pathways to other brain regions, involved in maternal behaviour and contribute to changes in maternal care and maternal anxiety, as seen after chronic stress exposure during the peripartum period (Maestripieri and D'Amato, 1991; Hillerer et al., 2011b; Purcell et al., 2011).

Finally, we assessed quiescence of stem cells, of which only limited knowledge of the functional significance of this process exists. We could show that the number of quiescent stem cells tends towards a decrease as a consequence of lactation and that chronic stress during postpartum abolishes this decrease with a concurrent decrease in the number of

proliferating stem cells. Importantly, stem cell quiescence was unchanged after chronic stress in virgin females, demonstrating the specificity of this stress effect to the peripartum period. Moreover, these findings strengthen the hypothesis of a changed cell cycle length, as decreased quiescence would lead to an accelerated cell cycle in stressed dams, due to a decreased availability of actively cycling stem cells. Moreover, they provide an explanation regarding the reduced survival observed following stress in lactation, as studies in hematopoietic stem cells revealed that increased levels of cell proliferation involve a higher frequency of replication errors (Arai et al., 2004; Hirao et al., 2004).

CONCLUSIONS

The results of the present study reveal important consequences of chronic stress during the peripartum period on different stages of adult hippocampal neurogenesis in the DG. The observed changes suggest that stress reverses the normal reduced proliferation and increased efficiency of neurogenesis in the peripartum period. These changes are likely to be mediated, at least in part, *via* increased apoptosis and a shortened cell cycle length, given the reduced neuronal differentiation and the increased quiescence of stem cells observed in the stressed group. Therefore, such alterations in neurogenesis may play a role in the behavioural and neuroendocrine adaptations observed following chronic lactation stress. Further research is required to elucidate specific regulatory mechanisms of cell proliferation, selection, differentiation and integration of newly generated cells.

Chapter 5

Prenatal *versus* postnatal stress: Differential effects on adult affective- and social-behaviour

Author's contribution:

Hillerer: Study design, performing of experiments, analyzing data, writing the manuscript

Lukas: Establishment of social preference test, performing experiments

Neumann: Study design

Slattery: Study design, contribution to manuscript writing

[Hillerer K.M., Lukas M., Neumann I.D., Slattery D.A. Prenatal *versus* postnatal stress: Differential effects on adult affective- and social-behaviour. Behavioural Brain Research, in preparation]

ABSTRACT

It is well-established that exposure to multiple episodes of early life stress significantly increase the risk for the development of mental illness in adulthood. Moreover, early mother-child interactions, including maternal care play a crucial role in the physiological and behavioural development of the offspring. However, to date, very few studies have directly compared the consequences of prenatal or postnatal stress on adult affective and social behaviour of the offspring and simultaneously assessed the impact of maternal behaviour on the behavioural outcome. In our study, we used a prenatal stress paradigm which consisted of stressing the dam between PD4 – PD16 by alternating RS and OC stress and postnatal stress, which was achieved by MS (120 min per day from LD2 - LD13). Furthermore, to compare more directly with the prenatal stress paradigm, an additional group of dams was subjected to RS during the separation phase. Both stress paradigms led to an increase in maternal behaviour directed towards the offspring but a decrease in body-weight gain in the stressed offspring. Prenatal stress resulted in an anxiogenic phenotype in adulthood, whereas postnatal stress had no effect on anxiety-related behaviour. In contrast, social preference was unaffected by prenatal stress, but abolished by MS; a deficit which was restored in the group where the dam was additionally stressed. Depressive-like behaviour as assessed in the FST was increased after prenatal stress, and postnatal stress, but only in MS+RS offspring. In summary, the results of the present study reveal that prenatal and postnatal stress has detrimental effects on the adult physiology and behaviour of the offspring and that they differentially alter adult affective and social behaviours. Moreover, they show that stress-induced changes in maternal care are able to restore at least some of the behavioural changes induced by early life stress.

INTRODUCTION

The time around birth and early childhood are known to be critical periods for shaping the adult phenotype in both rodents and humans. One reason for this is that significant developmental plasticity occurs in a variety of brain structures and systems, including the hippocampus, the amygdala and the prefrontal cortex (for reviews see Teicher et al., 2002; Bremner, 2003; Charil et al., 2010). Thus, not only in rodents, but also in humans disturbances by either pre- or postnatal stress critically alter these ongoing processes and, consequently, increase the likelihood of adult psychopathologies, such as anxiety and depression disorders, substance abuse and schizophrenia (Huttunen et al., 1994; Barnow et al., 2001; Heim and Nemeroff, 2001; Weinstock, 2001; Meaney et al., 2002; Howes et al., 2004; Fumagalli et al., 2007; Heim et al., 2008). While the outcomes of pre- and postnatal stress appear to strongly overlap, there are substantial species and sex-dependent differences (for review see Fumagalli et al., 2007).

Although, most human and rodent studies report a decreased birth weight after prenatal stress (Knackstedt et al., 2005; Rice et al., 2006; Mairesse et al., 2007; Seckl, 2008), there are studies in rodents showing no effect or even increased birth weight following prenatal stress (Keshet and Weinstock, 1995; Buhl et al., 2007). The discrepancies regarding body weight after prenatal stress also apply to studies on postnatal stress, showing either a decrease or an increase in body weight after MS (Kim et al., 2005; Matsumoto et al., 2006). The reported changes in body weight are accompanied by changes in the HPA axis; one of the key regulators of the stress response. During gestational stress, increased amounts of circulating glucocorticoids pass the placenta and, thereby, reach the foetus, leading to a long-term reprogramming of the HPA axis of the offspring, resulting in increased responsivity under

both basal and acute stress conditions (Vallee et al., 1997; Koehl et al., 1999; Lyons-Ruth et al., 2000; Ward et al., 2000; Maccari et al., 2003; Gutteling et al., 2005). Also after postnatal separation stress HPA axis function is consistently altered in rodents and humans. Such changes include an increase in basal CORT and ACTH levels, decreased CRH mRNA expression in the PVN and a decreased glucocorticoid receptor density in the hippocampus during adulthood (Aisa et al., 2008; Pesonen et al., 2010; Lajud et al., 2011). There is growing evidence that prenatal and postnatal stress implicate epigenetic mechanism, like epigenetic marking of the AVP gene that leads to an increased HPA axis activity (Darnaudery and Maccari, 2008; Murgatroyd and Spengler, 2011). Such an increase in HPA axis activity following pre-or postnatal stress results in developmental deficits in limbic structures, e.g. the amygdala, the PVN, the hippocampus and the prefrontal cortex (for reviews see Weinstock, 2001; Fumagalli et al., 2007; Seckl, 2008). Disruption of these limbic systems is a key component in the higher incidence of anxiety-related and depression-like behaviours observed following prenatal (Vallee et al., 1997; Ehlert et al., 2001; Dickerson et al., 2005; Estanislau and Morato, 2005; Brunton and Russell, 2010) and postnatal (Huot et al., 2001; Kalinichev et al., 2002; MacQueen et al., 2003; Romeo et al., 2003; Aisa et al., 2008) stress exposure. Although, there are studies revealing no effect on emotional reactivity after postnatal stress (Lehmann et al., 1999; Caldji et al., 2000; Hulshof et al., 2011), increased anxiety, fear and depression-like behaviour seem to be a robust and consistent finding after both prenatal and postnatal stress exposure in a variety of different test settings (Aisa et al., 2008; Darnaudery and Maccari, 2008; Kuramochi and Nakamura, 2009; Schulz et al., 2011). Beside the effects on adult affective behaviour, changes in social behaviours are well documented after early life stress. Thus, prenatal stress reduces juvenile play fighting and anogenital sniffing in male rats (Kleinhaus et al., 2010; Schulz et al., 2011), but moreover

significantly predicts the development of attention deficit hyperactivity disorders and autistic traits in humans (Ronald et al., 2010). Postnatal stress in the form of MS impairs social recognition in adult male rats (Lukas et al., 2010b), increases both adult intermale aggression and juvenile play-fighting (Veenema et al., 2006; Veenema and Neumann, 2009) and changes sexual behaviour, as seen in longer mount and transmission latencies (Rhees et al., 2001). Furthermore, maternally separated monkeys show abnormal social behaviours in the form of establishment of bonds with age mate peers and artificial surrogates (Suomi and Harlow, 1972). Interestingly, the quality of early mothering seems to play a pivotal role in restoring at least some of the effects of early life stress on adult behaviour in the offspring. This has been shown by elegant cross-fostering studies in mice bred for high or low anxiety behaviour (Kessler et al., 2011), but also in studies using stroking to mimic maternal anogenital stimulation during a separation period (Groer et al., 2002; Schmidt et al., 2002).

However, most of the studies, focusing on either, the effect of prenatal stress or postnatal stress, direct comparison of these two time-dependent stressors has not been performed. Moreover, the effect of the stress paradigms on the style of nursing behaviour is lacking so far. Therefore, we aimed to characterise the effects of prenatal (chronic pregnancy stress) and postnatal stress (MS) on the physiology and the behaviour of the offspring in one experimental outline.

MATERIAL AND METHODS

Animals

Female Wistar rats (Charles River, Sulzfeld, Germany; 200-250g) were housed in groups of four in standard polycarbonate rat cages and allowed to habituate for at least seven days. All rats were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00h, 22°C ± 1°C, 60 ± 5% humidity) and had free access to water and standard rat diet. All experimental procedures, except maternal observations and social behaviour tests (see below) were performed between 08:00 – 12:00h, approved by the Committee on Animal Health and Care of the local government of the Oberpfalz, and confirmed to international guidelines on ethical use of animals.

Mating procedure and confirmation of pregnancy

Two to three female rats were exposed to a male and pregnancy was verified by vaginal smears the following morning, which was designated PD0. All animals were returned to group cages with 4 females housed together (cage size 55 x 35 x 20 cm) until PD4 (prenatal stress paradigm) or PD16 (postnatal stress paradigm). From PD16 onward, all animals were single-housed. On the day of birth, i.e. LD1, all litters were culled to 8 (4 male, 4 female) pups.

Prenatal stress protocol

From PD4 to PD 16, dams were stressed *via* alternating daily RS (2 x 1 h at least 3 h apart during light phase; plexiglass column with ventilation holes; 12 cm diameter) and OC (4 unfamiliar rats for 24 h; cage size: 40 x 25 x 15 cm) periods. Rats were single-housed on RS days and taken from individual housing into OC conditions the following day. From PD16 the stressed rats were also kept in single-house conditions and left undisturbed until birth. Maternal care observations were carried out daily between LD2 and LD7, except on LD3, as described previously (Bosch and Neumann, 2008; Hillerer et al., 2011b). Body weight was assessed each day from PND2-PND8 and weekly from week 4 to week 12.

Postnatal stress protocol

Postnatal stress was conducted between PND2 and PND13 either in the form of MS (adapted from Veenema et al., 2006) or MS plus additional restraint stress of the lactating dams (MS+RS). Pups were separated from their dam for 2 h per day (10.00-12.00) and each litter was housed together, in an adjacent room, in a box filled with bedding, which was placed on a warm-blanket to maintain core body temperature. Dams were either left undisturbed in their home-cage (NS group and MS group) or stressed *via* RS for the 2 h of pup separation (for detailed descriptions see section prenatal stress procedure). After MS, pups were taken back in their home-cages and left undisturbed for the rest of the day. Maternal care observations were carried out daily between LD2 and LD13. According to a time-sampling protocol (Bosch and Neumann, 2008; Hillerer et al., 2011b) undisturbed maternal behaviour was observed for 5-10 s every second minute between 09:00h and 10:00h and from 12:00-14:00h. The pup-directed parameters assessed were ABN (quiescent kyphotic nursing),

blanket behaviour, laying on side or back. Moreover, we assessed licking and grooming pups; pup carrying, digging, locomotion, rearing, eating/drinking, self-grooming and sleeping. The frequency of the recorded behaviour, e.g. ABN, was calculated as the mean total ABN per day. Body weight was assessed each day from PND2-PND8 and weekly from week 4 to week 12.

Behavioural tests

Behavioural tests were performed in adult offspring (PND70 onwards), with an interval of seven days between the tests to minimize any stressful effects from the previous test(s).

Social preference test (SPT)

At the age of 10 weeks the social preference was tested with the SPT. The test was performed during the active phase, 1 hour after lights out (19.00-22.00) under red light as described previously for rats (Lukas et al., 2011). Briefly, the experimental rat was placed in a novel arena (40 x 80 x 40 cm). After 30 s of habituation, an empty-wire-mesh cage (non-social stimulus; 20 x 9 x 19 cm) was placed at one side wall of the arena for 4 min. The empty cage was then exchanged by an identical cage containing an unknown juvenile rat of the same sex (social stimulus) for additional 4 min. Before each trial, the area was cleaned with water containing a low concentration of detergent. The test procedure was videotaped and scored afterwards by an observer blind to treatment using JWatcher behavioural observation software (V 1.0, Macquarie University and UCLA). Non-social stimulus and social stimulus investigation times were scored by measuring the time the rat spent in active

olfactory investigation. A significantly higher time of investigation of social *versus* the non-social stimulus was considered social preference.

Light-dark box (LDB)

The LDB was performed in 11 week old prenatally stressed offspring in order to assess the anxiety-related behaviour as described previously (Waldherr and Neumann, 2007; Slattery and Neumann, 2010). Briefly, the LDB setup consisted of one lit (40 x 50 cm, 350 lux) and one dark compartment (40 x 30 cm, 70 lux) connected *via* a small opening for transition (7.5 x 7.5 cm). The floors were divided into squares (10 x 10 cm). Rats were placed in the dark compartment, line-crossings, time spent in each compartment and rearings in the light compartment during the 5-min test were assessed on-line *via* a camera located above the box.

Elevated plus- maze (EPM)

The EPM was performed in postnatally stressed offspring, in order to assess the anxiety-related behaviour in week 11 as described previously (Pellow et al., 1985; Wall and Messier, 2001). Briefly, the maze consisted of two open and two closed arms (50 x 10 cm) arranged in a cross from the neutral zone (a 10 x 10 cm square) and was elevated 80 cm from the ground. Rats were placed in the closed arm, and entries and time spent in each compartment during the 5-min test were assessed on-line *via* a camera located above the box.

Forced swim test (FST)

The FST was conducted in order to assess the depression-like behaviour of the offspring in week 12 (adapted by Slattery et al., 2005). Briefly, rats were individually placed into a plexiglass cylinder (30 x 49 cm) filled with 25 ± 1°C water to a depth of 30 cm. The rats were removed after 15 min, towel-dried, and returned to their home cage. Water was changed between each test. Twenty-four hours after this first exposure, the rats were replaced in the swim cylinder for 5 min. Each swim session was video-taped for subsequent analysis. Using a time sampling technique, the predominant behaviours, climbing, swimming or immobility, were assessed by an observer blind to treatment in each 5-s period of the first 5 min of the first exposure, and of the full 5 min of the 2^{nd} swim exposure (providing an overall total of 60 scores for each day). Climbing behaviour consisted of upward-directed movement of the forepaws usually along the side of the swim cylinder, swimming behaviour consisted of horizontal movement, and immobility was defined as floating in the water only making movements necessary to maintain its head above the water (for pictorial representation of the behaviours see Cryan et al., 2002)

Statistics

Results were analysed using either an unpaired Student's t-test, Mann Whitney *U*-test, or a two way analysis of variance (ANOVA) with or without repeated measures, as appropriate. Any statistical differences, which were set at $p \leq 0.05$, were further analysed using a Fisher's *post-hoc* test. Data are expressed as mean ± S.E.M. Statistical analyses were performed using SPSS for Windows (version 16; SPSS Inc, Chicago, IL, USA).

RESULTS

Chronic stress effect on maternal behaviour

Repeated-measures ANOVA revealed that ABN across the hours assessed in the lactation-stress paradigm differed across the groups ($F_{1,23}$=15.248; p=0.001). *Post-hoc* analysis revealed that the dams subjected to RS during lactation showed an increase in ABN on LD9, LD12 and LD13 compared with both the non-stressed (NS) group ($p < 0.05$; Fig. 18) and the MS group on LD3 and from LD10 to LD13 ($p < 0.05$; Fig. 18). However, pup separation alone did not lead to an alteration in ABN compared with controls on any day assessed. No effect was observed in ABN on other days or any other maternal behaviour assessed (data not shown).

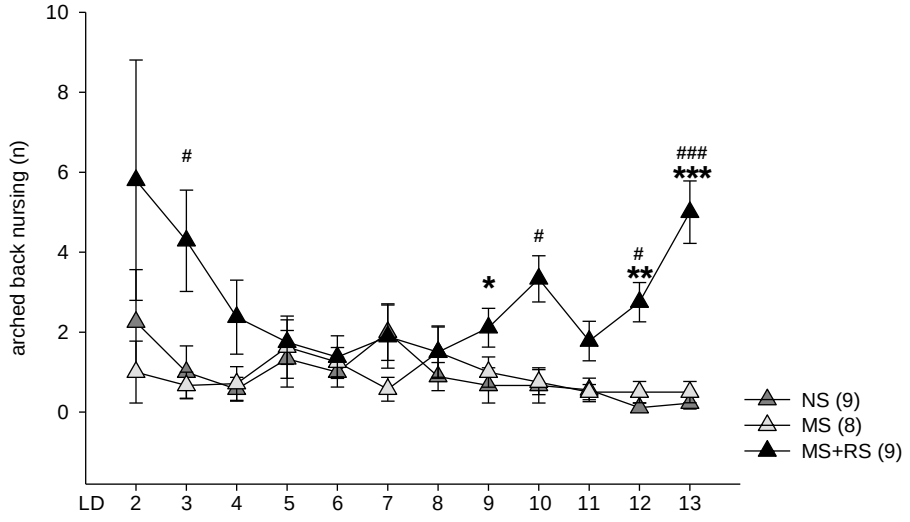

Figure 18: Effect of stress and pup separation on maternal behaviour

Maternal behaviour was assessed from LD2-LD13 one hour before and two hours after MS/MS+RS. MS+RS resulted in increased ABN, whereas MS alone did not affect active maternal care. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, p<0.05 *vs.* NS;**, p<0.01 *vs.* NS; ***, p≤0.001 *vs.* NS; # p<0.05 *vs.* MS; ###, p<0.001 *vs.* MS; NS, non-stressed, MS, maternal separation; MS+RS, maternal separation plus restraint stress of the dam; LD, lactation day

Effect of early life stress on body weight gain

There was a significant weight difference from week 4 to week 12 between prenatally stressed (S) *versus* non-stressed (NS) male offspring ($F_{1,12}$=9.575; p=0.007). *Post-hoc* analysis showed lower body weight gain in S males in each week analysed (p<0.05; Fig. 19A). Postnatal stress had no effect on weight development between PND2 and PND8 ($F_{1,22}$=0.248; p=0.783; data not shown). While no main effect was observed on weight gain between weeks 4-12 ($F_{1,22}$=1.676; p=0.22), separate Mann Whitney *U* analyses revealed that both MS and MS+RS led to reduced body weight gain in weeks 11 and 12 compared with control offspring (p<0.05; Fig. 19B).

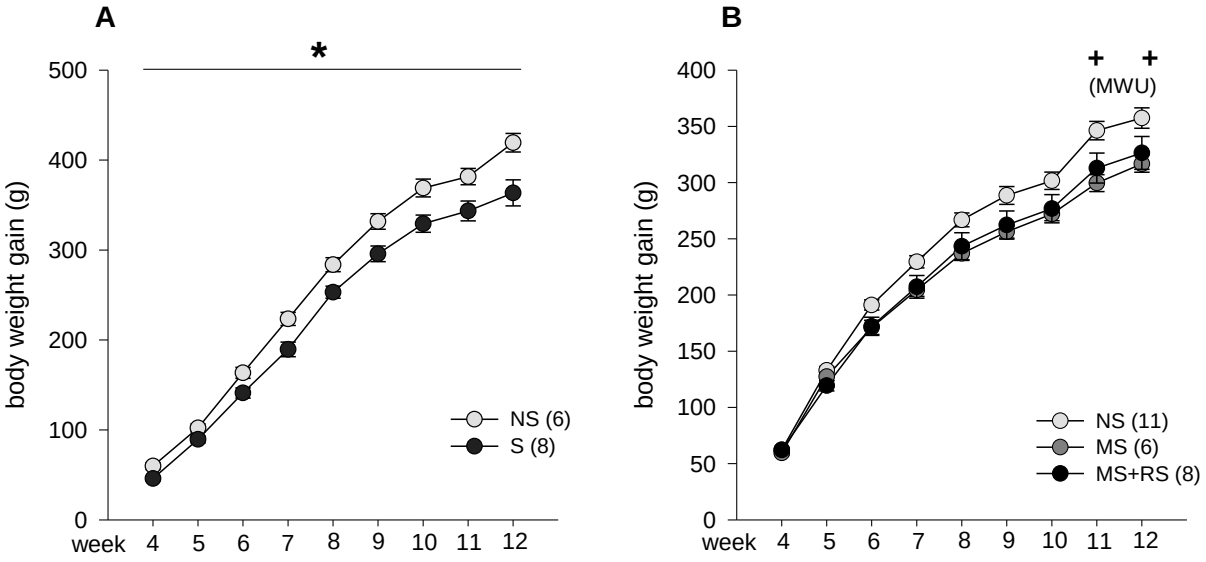

Figure 19: Effect of early life stress on body weight gain

Body weight gain was assessed weekly between week 4 and week 12 in prenatally and postnatally stressed offspring. Exposure to prenatal (A) or postnatal (B) stress led to an impaired body weight gain. A) Prenatally stressed offspring displayed a decreased body weight gain from week 4 onwards. B) Rats exposed to both forms of postnatal stress showed a decrease in body weight gain in week 11 and week 12. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, p<0.05 *vs.* NS; ++, p<0.05 *vs.* NS using a Mann Whitney *U* test (MWU); NS, non-stressed; S, stressed (restraint stress of the dam) MS, maternal separation; MS+RS, maternal separation plus restraint stress of the dam

Effect of early life stress on social behaviour

A two way ANOVA for repeated measures (stress x stimulus) revealed that stress during the prenatal period did not affect social preference (factor stress: $F_{1,43}$= 0.856; p=0.379; factor stimulus: $F_{1,43}$=110.765; p≤0.001). Thus, both NS and S groups demonstrated a preference for the social, compared to the non-social stimulus in the SPT (p≤0.001). Moreover, the investigation time of the social stimulus did not differ between S and NS rats (Fig. 20A). However there was a significant interaction effect on social preference after postnatal stress ($F_{1,22}$=4.858; p=0.018). *Post-hoc* analyses showed that only NS and MS+RS rats demonstrated a preference for the social, compared to the non-social stimulus (Fig. 20B). Furthermore, MS rats spent significantly less time with the social stimulus, compared to NS rats (p<0.05; Fig. 20B). There was no difference in the time spent with the non social stimulus (cage) between the different stress groups (Fig. 20B).

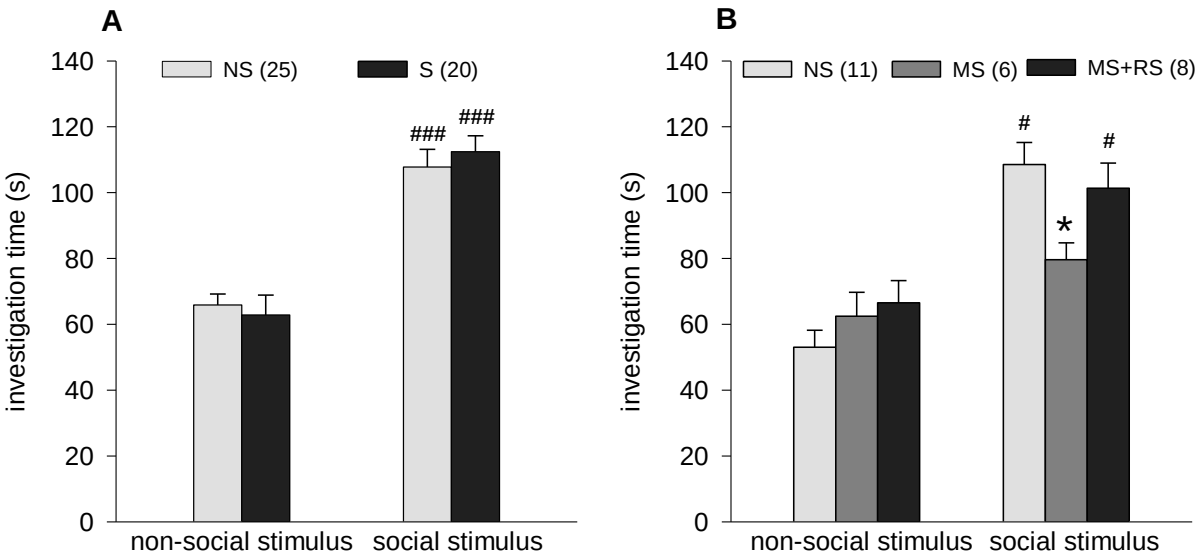

Figure 20: Effect of early life stress on social preference

Social preference was assessed in prenatally (A) or postnatally (B) stressed offspring at an age of ten weeks, using the SPT. A) Prenatal stress had no effect on social preference, as seen in an equal amount of time spent with the non-social or the social stimulus in NS and S rats. B) MS led to a reduced social preference as seen in a reduced time spent with the social stimulus, when compared with NS rats; MS+RS did not differ in social preference compared to NS rats. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.05$ *vs.* NS; #, $p<0.05$ *vs.* non-social stimulus; ###, $p\leq0.001$ *vs.* non-social stimulus; NS, non-stressed; S, stressed (restraint stress of the dam) MS, maternal separation; MS+RS, maternal separation plus restraint stress of the dam

Effect of early life stress on anxiety-related behaviour

Prenatal stress increased anxiety-related behaviour in the LDB. Independent sample t-test revealed a reduced percentage time spent in the light compartment (box) of the LDB ($p=0.039$; Fig. 21A), as well as a reduction in the number of rearings in the light compartment ($p=0.038$; Fig. 21A) and the dark compartment ($p=0.041$; Fig. 21A) of the LDB. The number of line-crossings in the dark compartment, indicative for locomotor activity, did

not differ in between the groups (data not shown). However, neither postnatally stressed group differed in anxiety-related behaviour from controls as assessed by the EPM (Fig. 21B).

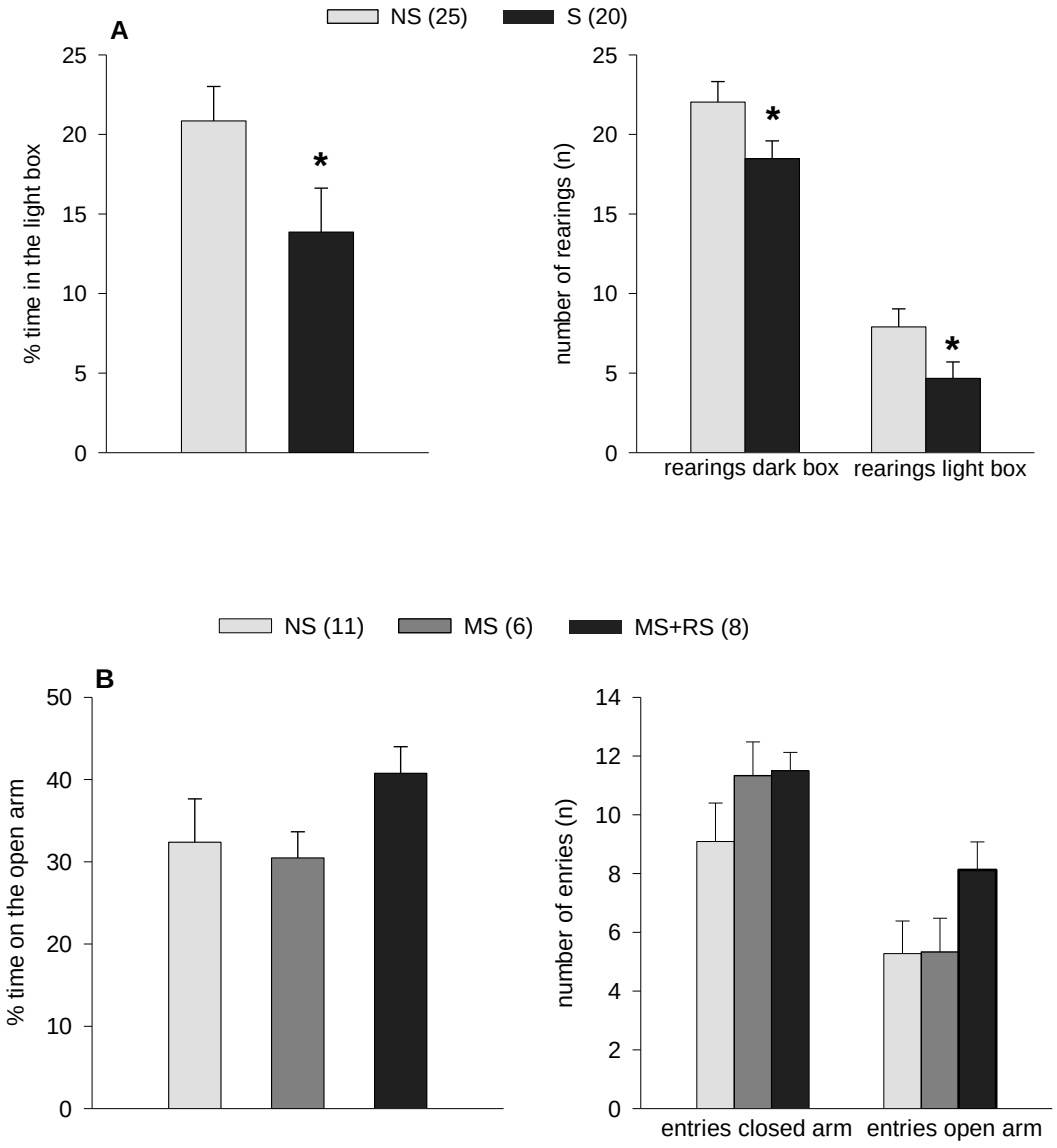

Figure 21: Effect of early life stress on anxiety-related behaviour

Anxiety-related behaviour was assessed in prenatally (A) or postnatally (B) stressed offspring at an age of eleven weeks, using the LDB (A) or the EPM (B). A) Prenatal stress increased anxiety-related behaviour as seen in a reduced percentage time spent in the light compartment (box) of the LDB and a lower number of rearings in the light and dark compartment of the LDB, when compared with the NS group. B) Postnatal stress did not affect anxiety-related behaviour on the EPM; neither MS, nor MS+RS differed from NS rats in the percentage time spent on the open arms of the maze or in the number of entries in open and closed arms of the maze. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, $p<0.05$ *vs.* respective NS group; NS, non-stressed; S, Stressed (restraint stress of the dam) MS, maternal separation; MS+RS, maternal separation plus restraint stress of the dam

Effect of early life stress on depression-like behaviour

A significant effect of prenatal stress was observed on climbing ($F_{1,12}$= 24.016; p<0.001) and immobility ($F_{1,12}$= 35.164; p=0.001) behaviour, but not on swimming ($F_{1,12}$= 0.014; p=0.909). *Post-hoc* analyses revealed that stress increased climbing with a concurrent decrease in immobility compared to equivalent NS group (p<0.001; Fig. 22 A). Postnatal stress did not significantly affect depression-like behaviour in the FST. However, separate analyses confirmed that MS+RS climbed less and floated more, when compared with NS and MS rats (Mann Whitney *U*; p<0.05: Fig. 22B).

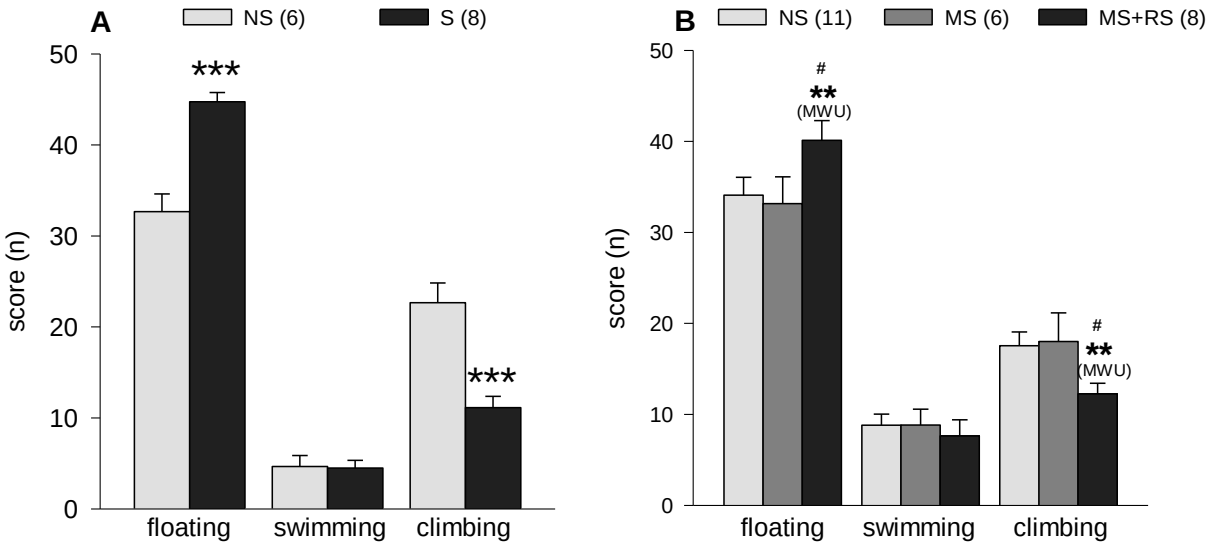

Figure 22: Effect of early life stress on depression-like behaviour

Depression-like behaviour was assessed in prenatally (A) or postnatally (B) stressed offspring at an age of twelve weeks, using the FST. A) S animals climbed less and were more immobile than NS rats. B) MS alone did not affect FST behaviour, however, MS+RS led to a decrease in floating with a concurrent decrease in climbing, when compared with NS or MS rats. Data represent mean ± SEM with the numbers in parentheses representing the group sizes. ***, p<0.001 *vs.* respective NS; **, p<0.01 *vs.* respective NS using a Mann Whitney *U* test; #, p<0.05 *vs.* MS using a Mann Whitney *U* test. NS, non-stressed; S, stressed (restraint stress of the dam) MS, maternal separation; MS+RS, maternal separation plus restraint stress of the dam

DISCUSSION

The aim of the present study was to directly compare the effects of prenatal *versus* postnatal stress on adult affective and social behaviours, in dependence on alterations in maternal behaviour that were induced by chronic peripartum stress. Both prenatal and postnatal stress led to a decreased developmental body weight gain in the offspring, which persisted from weaning to adulthood. Prenatal stress induced an anxiety-related and depression-like phenotype, whereas MS resulted in a change of social behaviours without changes in adult affective behaviour. However, MS+RS increased depression-like behaviour in the offspring, but restored impaired social preference after postnatal stress. These differences may be related to the increased nursing induced by the additional restraint of the dam. Thus, prenatal and postnatal stress affects adult affective and social behaviours in a different manner and the quality of maternal behaviour appears to be an important factor that contributes to the development of such behaviours. Future studies will determine the effect of cross-fostering the pups exposed to gestational stress to determine whether the behavioural and physiological consequences are modulated by the different nursing levels.

Body weight gain is decreased despite an increase in daily ABN

Consistent with previous studies revealing an effect of chronic stress during pregnancy on ABN and licking and grooming behaviour (Champagne and Meaney, 2006; Hillerer et al., 2011b), we could show that chronic stress during lactation leads to an increase of ABN in mid-lactation (LD9-13). Despite the observed increase in active maternal care, body weight gain was decreased in both prenatally and postnatally stressed offspring, as consistently

reported (Lesage et al., 2004; Buhl et al., 2007; Darnaudery and Maccari, 2008; Emack et al., 2008; Hillerer et al., 2011b). Interestingly, while in the prenatally stressed offspring the reduction of body weight gain appeared within the first days after birth and persisted until adulthood, in postnatally stressed offspring this reduction primarily occurred in adulthood. The decreased body weight gain observed in prenatally stressed offspring might be mainly due to the harming effects of glucocorticoids *in utero*. Chronic pregnancy stress has previously been shown to lower the expression of the placental glucocorticoid "barrier" enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Mairesse et al., 2007) and CBG plasma levels, which both increase the concentration of glucocorticoids reaching the foetus. In contrast, the amounts of CORT reaching the offspring *via* the milk during lactation are relatively low but might be enough to affect the physiology of the offspring. Moreover, it has to be considered that postnatal stress occurs within the time frame of the stress hyporesponsive period (SHRP), when the neonatal rat is less sensitive to the stimulatory effects of stress, but also the inhibitory effects of circulating CORT (for review see Sapolsky and Meaney, 1986). Thus, disruption of mother-pup interaction in early life is likely to increase CORT in the pups, which might alter developing systems and thereby have long-lasting effects on the behavioural and physiological outcome in adulthood, but might not immediately have an effect on the physiological development of the offspring, which might moreover explain the later onset of body weight gain changes in the postnatally stressed offspring. However, it has also to be kept in mind that the amount of milk ejected and its content, i.e. fat content could be affected by both pregnancy and lactational stress paradigms, which would also play an important role in offspring weight development. Therefore, future studies are needed to determine a possible influence of those parameters on body weight gain changes.

Effect of prenatal and postnatal stress on adult affective and social behaviour and the role of changes in maternal behaviour

In the present study, we could show that prenatal and postnatal stress differentially affects anxiety-related and depression-like, as well as social behaviours. While prenatal stress tended towards changes in adult anxiety-related and depression-like behaviours, postnatal stress rather affected social abilities of the offspring. The observed discrepancies in the behavioural outcome might be due to the timing of the stressor during brain development. In animals that give birth to relatively mature neonates, most of the neuroendocrine maturations occur *in utero* (Lupien et al., 2009). Hence, it is not surprising that stress during this sensitive stage of brain development has detrimental effects on some important circuits, which have the potential to induce alterations in behaviour. The increase in anxiety-related behaviour, seen in the prenatally stressed group is a well known consequence of prenatal stress in rats and mice (Fride and Weinstock, 1988; Vallee et al., 1997; Ward et al., 2000; Nishio et al., 2001; Patin et al., 2004; Rice et al., 2007; Darnaudery and Maccari, 2008; Miyagawa et al., 2011) and might be due to the effects of stress on the developing CRH system of the amygdala, which has been shown to mediate the cognitive and physiological expression of fear and anxiety. Several studies revealed that the fetal CRH system of the amygdala becomes responsive during the last week of gestation (for review see Weinstock, 2008) and that prenatal stress affects the development of this system (Cratty et al., 1995; Vallee et al., 1997; Dickerson et al., 2005; Estanislau and Morato, 2005; Zohar and Weinstock, 2011). Furthermore, an involvement of the OXT system in the PVN has to be considered. The PVN develops from PD13 to PD15 onwards and is able to react on maternal glucocorticoids from this stage onward (Fujioka et al., 1999; Fujioka et al., 2003). It can be speculated, that prenatal stress affects the PVN, which is an important region not only for

stress responsivity, but for anxiety (Neumann, 2007; Blume et al., 2008). Therefore, future studies will assess the effect of prenatal stress on the expression levels of CRH, as well as important hypothalamic targets, such as OXT and AVP, and their respective receptors, which have been shown to have opposing effects on anxiety-related behaviour (Landgraf and Neumann, 2004). Given the known comorbidity between anxiety and depression (Hinojosa et al., 2006; Micco et al., 2009), the increase in depression-like behaviour seen after prenatal stress, is in accordance with the observed changes in anxiety and also fit with previous studies showing an increased depression-like behaviour after prenatal stress exposure (Vallee et al., 1997; Smith et al., 2004).

Interestingly, neither postnatal stress paradigm affected anxiety-related or depression-like behaviour. Although alterations in such behaviours following early life stress have been reported (Wigger and Neumann, 1999; Huot et al., 2001; MacQueen et al., 2003; Romeo et al., 2003; Aisa et al., 2008), these findings are not consistent (Caldji et al., 2000; Hulshof et al., 2011). This suggests that longer separation periods are needed to induce robust effects on anxiety-related and depression-like behaviour after postnatal stress. Despite no change in adult affective behaviours postnatal stress led to an impaired social behaviour, which is consistent with previous work. Accordingly, MS leads to reduced juvenile play fighting and anogenital sniffing (Veenema and Neumann, 2009; Kleinhaus et al., 2010; Schulz et al., 2011) and impaired social recognition in rats (Lukas et al., 2010b), as well as reduced contact seeking in monkeys (Dettling et al., 2002; Feng et al., 2011). The observed changes might be due to abnormalities in brain regions undergoing particularly early postnatal rather than prenatal plasticity and development, like the lateral septum, the hippocampus and the prefrontal cortex (Bremner et al., 1997; Huot et al., 2001; De Bellis et al., 2002). Thus, postnatal stress alters important neural circuits underlying social behaviour, such as OXT and

AVP systems of the lateral septum and the piriform cortex. In detail, MS decreases the expression of the OXT-R in the lateral septum and leads to an increase in vasopressin V (1A) receptor (V1a-R) expression in the piriform cortex (Lukas et al., 2010a). Moreover, impairments in social recognition after MS are accompanied by a blunted response of AVP (Lukas et al., 2010b). It might be hypothesized that similar alterations underlie the observed changes in social behaviour in this study. Therefore, future studies will assess the effect of MS on systems described above.

Alternatively, the deficits in adult social behaviour may result from disruption of social setting in early life, which would also explain why the rats exposed to prenatal stress in the present study did not display deficits in social preference behaviour.

Interestingly, while MS alone did not affect anxiety-related or depression-like behaviour, additional stress of the dam led to a slight trend towards a reduced anxiety and an increased depression-like behaviour in the adult offspring. Similarly, whereas social preference was decreased in MS animals, MS+RS rats did not differ from unstressed control animals. These results together with the observed alterations in maternal care raise the question whether an increase in nursing is able to restore the effects of early life stress on the adult behaviour. It is well documented in the literature that variations in maternal care represent an important factor for the adult behavioural outcome (Liu et al., 1997; Caldji et al., 1998; Meaney, 2001; Pryce et al., 2001). Natural variations in maternal care and mother-infant bonding are known to change sensorimotor gating (Zhang et al., 2005), cognition and learning, as well as fear in rats (Liu et al., 1997; Caldji et al., 1998; Liu et al., 2000) and are fundamental for the development of social skills, including social bonds in primates (Suomi, 1997). Although, increased amounts of ABN were able to restore the deficits in social behaviour induced by MS, they did not significantly affect the outcome in anxiety-related

and depression-like behaviour. Therefore, it seems that anxiety and depression changes are very common and robust effects of early life stress exposure, whereas social deficits seem to be rather dependent on the timing of the stressor and may be rescued by high amounts of maternal care perceived during the early postnatal period. Furthermore, future studies will assess the impact of cross-fostering in the prenatal stress paradigm, as we have previously shown that stressed dams display a higher frequency of ABN (Hillerer et al., 2011b). Hence, it might be that the increased nursing received by the offspring of gestational stressed dams alters the behavioural outcome in adulthood. To investigate this possibility, we will determine the effects of increased nursing on control pups cross-fostered to stressed dams and reduced nursing in stressed offspring raised by control dams. Importantly, previous studies in mice have already shown an association between high amounts of nursing received and the level of anxiety in adulthood. Thus, when HAB mice, which also receive increased levels of ABN, were cross-fostered to less attentive LAB dams, they displayed reduced anxiety in adulthood (Kessler et al., 2011).

CONCLUSIONS

Chronic stress during early life, i.e during the prenatal or postnatal period, has profound effects on the developing neonatal brain and therefore constitutes an important risk factor for the development of psychopathologies in adulthood. By directly comparing prenatal and postnatal stress, we could demonstrate that stress that occurs *in utero* leads to a change in adult anxiety-related and depression-like behaviour, while stress early after birth interferes

with the development of normal social behaviour. The behavioural abnormalities in the early life stress paradigm could be partially rescued by increased maternal care, suggesting that mother-infant interactions could play a crucial role in the development of mental diseases after early life stress exposure. Thus, cross-fostering studies will be performed in the prenatal stress paradigm to determine whether the received nursing can shape adult behaviour following gestational stress. Future studies will be required to identify the neuronal parameters associated with the observed behavioural abnormalities and thereby help to shed light on the mechanisms underlying adult affective and social disorders after adverse early life events.

Chapter 6

General Discussion

Content

1. Summary of results

The goal of the present study was to assess the impact of chronic stress during pregnancy on peripartum-associated changes (chapter 2 and 4). In order to achieve this aim, it is necessary to determine whether such changes are specific to the peripartum period (chapters 2-4). This is especially important for two reasons: 1) The peripartum period is a time of stress hyporesponsiveness and, thus, may not cause the same degree of changes as in virgin females. 2) To gain a better insight into peripartum stress-related disorders, which are characterised by their very specific time of onset during pregnancy. Such findings, as outlined below, increase our understanding of the aetiology of these serious disorders. Moreover, the influence these changes have on the offspring can be investigated to determine preventative measures that can be pursued to minimize the physiological and behavioural pathologies, which occur in their adulthood (chapter 5)

The peripartum period is a characterised by numerous neuroendocrine, molecular and behavioural adaptations. Thus, while these adaptations encompass important mechanisms that prepare the female for motherhood, disruption of them, for example by chronic stress during pregnancy, has been implicated in the development of postpartum mood and anxiety disorders. However, there is currently a lack of appropriate models and studies assessing the effects of chronic stress on the above mentioned adaptations (see chapter 1 and 2 for details). Therefore, in chapter 2, I established a model of chronic stress during pregnancy, which I have used to study the effects of stress on a variety of known peripartum adaptations at different levels. Using this paradigm, I was able to demonstrate that chronic stress exposure prevented a host of important changes normally seen during pregnancy and

lactation. In more detail, I could reveal that pregnancy stress prevented the basal hypercorticism, the increased OXT mRNA expression in the PVN and the anxiolysis associated with the peripartum period. However, chronic stress neither affected the peripartum-associated decrease in CRH mRNA expression, the attenuated CORT response to an acute stressor, nor did it affect hypothalamic AVP mRNA expression. Moreover, I could demonstrate that the increased anxiety-related behaviour was concomitant with an increase in active maternal care, whereas there was no correlation with depression-like behaviour in the FST, which was unchanged by chronic stress exposure. By additionally assessing the effect of acute treatment of the dams with imipramine, which acts predominantly on the noradrenergic system, I could show that chronic stress reversed the behavioural response to this antidepressant. Thus, while imipramine reduced the peripartum-associated passive behaviour in non-stressed dams, it had an opposite effect in stressed dams. This suggests that stress exposure during pregnancy prevents the peripartum associated attenuation of the noradrenergic system.

In chapter 3, I demonstrated that males and females differ in adult hippocampal neurogenesis under both basal and chronic stress conditions. Moreover, I showed that CORT levels were also affected in a sex-specific manner suggesting that they, at least in part, mediate some of the sex differences in neurogenesis. In detail, cell proliferation was reduced after chronic stress in males, which was reflected by an increase in stem cell quiescence and a concurrent decrease in proliferating stem cells. While females seemed to be unaffected by stress exposure in terms of cell proliferation and stem cell quiescence, they showed a robust decrease in cell survival. Although, cell survival was lower in males under basal conditions, there was no further reduction observed after chronic stress. Sex differences were also

observed in the number of immature neurons, with males expressing more immature neurons compared to females. Although, immature neurons have been implicated in spatial working memory, males and females did not differ in a spatial working memory task. Even though the analysis of astroglial and neuronal differentiation patterns revealed no basal sex differences, it showed that chronic stress reduced the percentage of mature neurons in females, but not males, without changes in the percentage of astroglial cells in either sex. Beside the sex-specific differences in adult hippocampal neurogenesis, I provided evidence for a higher basal CORT level in females compared to males, whereas the exposure to chronic stress did not affect CORT levels in either sex. The fact that males and females differ their HPA axis activity, suggests that this might be one of the mechanism regulating the observed sex differences in adult hippocampal neurogenesis.

Based on the results of chapter 3, in the following chapter 4, I was able to show that chronic stress during the stress hyporesponsive period of lactation affects common peripartum-adaptations at the level of the hippocampus. Confirming the reproducibility and validity of my model, I replicated the findings of previous studies, showing a lactation-induced decrease in cell proliferation and cell survival in the DG. These findings were first and foremost used as a positive control, which enabled me to establish the novel findings that differentiation patterns and stem cell quiescence are altered by chronic stress during lactation. Thus, I revealed that chronic stress during lactation reversed the peripartum-associated decrease in cell proliferation, although the number of proliferating stem cells was decreased in stressed animals. Similar to the results in virgins, chronic stress during lactation had no effect on the number of immature neurons and astroglial differentiation, whereas neuronal differentiation was impaired. Basal CORT levels were shown to be increased in

stressed dams, which would be expected to decrease neurogenesis processes. Hence, given the observed changes in cell proliferation, differentiation and quiescence, the latter result suggest that there must be other mechanisms beside CORT that regulate adult hippocampal neurogenesis in lactation under basal and chronic stress conditions.

In chapter 5, I could demonstrate that the timing of early life stress and the amount of nursing received differentially affects adult affective and social behaviour of the offspring. Thus, I was able to show that prenatal stress induces an anxiety-related and depression-like phenotype, while postnatal stress led to diminished social behaviour. Moreover, I revealed that chronic stress during the peripartum period increases active maternal behaviour. Importantly, the increased ABN was able to mitigate the deficits in social behaviour induced by MS. However, it was ineffective to restore abnormalities in adult affective behaviour seen after prenatal stress and even worsened depressive-like behaviour after MS.

In summary, I provided evidence that chronic stress has detrimental effects on neuroendocrine, molecular and behavioural parameters. Not only could I show that stress affects males and females in a sex-dependent manner, but moreover that the effects of chronic stress are dependent on the reproductive status. The performed studies contribute to a better understanding of the aetiology of postpartum mental disorders and the observed consequences in the offspring. Therefore, the systems identified to be altered by peripartum stress may represent appropriate biomarkers or therapeutic targets for postpartum mood and anxiety disorders. Moreover, they show the importance of such changes in the behavioural and physiological consequences for the offspring.

2. Effects of chronic stress on peripartum-associated adaptations

Despite the fact that women are twice as likely to develop stress-related disorders, most basic studies assessing the detrimental effects of chronic stress are performed in males. This is due, in part, to the complexity of studying chronic stress in females, given the differences in a number of factors, including OXT-R expression (Bale and Dorsa, 1995) and HPA axis activity (Atkinson and Waddell, 1997) across the oestrus cycle. Further, it is well known that males and females exhibit different stress sensitivity and coping mechanisms (Bowman et al., 2001; Wolf et al., 2001; Dalla et al., 2009) and, therefore, stressors that are effective in males may not be in females and *vice versa*. Furthermore, the choice of an appropriate stressor in females is complicated under situations when the stress response, and various other parameters, are naturally altered, as it occurs during the peripartum period. Moreover, it is generally accepted that social-based stressors more accurately reflect the human situation than purely physical stressors and, thus, are more desirable in novel paradigms (Cryan and Slattery, 2007).

Given the fact that the time around birth is characterised by an increased risk to develop postpartum mood and anxiety disorders, and chronic stress has been shown to be one prominent risk factor underlying the development of such mental disorders, assessing the effect of chronic stress on peripartum adaptations with appropriate animal models is of vital importance. The majority of such studies performed to date actually use pregnancy stress as a form of early-life stress and do not determine the consequences on the mother. Additionally, before using an animal model to assess the effect of stress on peripartum adaptations, it is required to assess the impact of chronic stress in male, but particularly in virgin animals to determine the specificity, or not, of the changes to the peripartum period.

Therefore, in all of my studies, the effects of chronic stress during either pregnancy or lactation were compared with those in either virgin, or virgin and male rats.

2.1 Choice and validation of the stress paradigms employed in the present studies

In order to induce a psychosocial-based stress paradigm, I alternated daily RS (2 x 1 h) and OC (24 h) from PD4 to PD16, whereas daily 2 h RS from LD2 to LD13 was used to induce chronic stress in lactating dams. The addition of a social component to the physiological stressor of restraint was to complement the effect of RS due to the peripartum-associated stress hyporesponsivity (Brunton et al., 2008; Slattery and Neumann, 2008). Although, using overcrowding as an additional social stressor in lactating dams would have facilitated a more direct comparison between chronic pregnancy stress and lactation stress, it is not feasible in lactation due to the presence of pups, which also leads to high aggression levels of the dams (Deschamps et al., 2003; Bosch et al., 2005; Bosch and Neumann, 2010), and was therefore not included in the paradigm. However, both RS and OC stress are effective stressors in female rats also when administered alone (Brown and Grunberg, 1995; Baranyi et al., 2005; Neumann et al., 2005a). Validating the chronic stress paradigms, I showed that chronic stress during pregnancy or lactation leads to a decrease in body weight gain and alterations in CORT levels (see below for more discussion). Moreover, dams exposed to stress during pregnancy showed an increase in adrenal weight and a decrease in thymus weight. These results are in accordance with studies in male mice (Reber et al., 2006; Reber and Neumann, 2008) and rats (Bielajew et al., 2002; Baranyi et al., 2005; Qi et al., 2006; Marin et al., 2007; Rygula et al., 2008; Herzog et al., 2009) showing an effect of chronic stress on above mentioned physiological parameters.

2.2 Consequences of stress on behavioural and neuroendocrine adaptations

As previously stated, lactation is associated with a basal hypercorticism / hypercortisolism (Stern et al., 1973; Altemus et al., 1995; Walker et al., 1995; Windle et al., 1997; Lightman et al., 2001; Taylor et al., 2009) with a concurrent attenuation of the HPA axis response to acute stressors in both rodents and humans (Neumann et al., 1998b). While chronic stress during pregnancy did not affect the attenuated CORT response to an acute 60 s swim stress, it prevented the lactation-associated hypercorticism. Importantly, neither parameter was affected in the virgin animals, which suggests that the reduced plasma level of CORT is specific to the peripartum period. Although lactation has also been associated with an attenuated rise of ACTH to an acute stressor, I did not observe such an effect in non-stressed or stressed dams. The fact that the adrenals were enlarged but basal plasma CORT levels were lower in stressed dams compared to non-stressed dams implies that adrenal insufficiency is a consequence of the stress exposure. These results are in agreement with studies in male mice revealing a similar effect at the adrenal level after chronic psychosocial stress (Reber et al., 2007). Importantly, they allow drawing a link to human studies, where changes in peripartum neuroendocrine patterns have been associated with various postpartum psychopathologies. Thus, decreased awakening cortisol levels have been observed in mothers who went on to develop postpartum depression (Taylor et al., 2009). In contrast, women with postpartum thoughts of harming the infant have been shown to display increased morning ACTH hormone levels, without any changes in cortisol levels (Labad et al., 2011) and elevated concentrations of CRH in the CSF have been found in rhesus macaques females that abuse their children (Maestripieri et al., 2005). Consequently, it seems that differences in neuroendocrine patterns are strongly dependent on the type of postpartum psychopathology. Therefore, disorders associated with postpartum thoughts of

harming the infant seem to share main features of obsessive-compulsive disorders (Kluge et al., 2007; Labad et al., 2011), while hypocortisolism is comparable to characteristics in patients suffering from posttraumatic stress disorders, chronic fatigue syndrome or postpartum depression (Kammerer et al., 2009; Taylor et al., 2009). Furthermore, the lower plasma levels in stressed dams in the present studies were observed at the beginning of the light phase and, thus, it would be interesting, and even necessary, to determine whether the levels are also lower at the start of the dark phase, i.e. when CORT levels rise in rodents. This is all the more necessary as there was no effect of chronic stress on depression-like behaviour in the FST. However, it is important to mention that noradrenergic inputs from the brainstem, as well as adrenergic receptor expression in the PVN are downregulated during the postpartum period, which contributes to the decreased stress responsiveness (Douglas et al., 2005). These changes might contribute to the decreased climbing behaviour in non-stressed lactating dams, because climbing behaviour has been shown to be mediated by catecholamines (Cryan and Slattery, 2010). Treatment with the antidepressant imipramine generally increases climbing behaviour, as it acts predominantly on the noradrengic system. Consequently, acute treatment with imipramine led to an increase in climbing behaviour with a concomitant decrease in immobility in non-stressed dams. In contrast, stressed dams treated acutely with imipramine displayed decreased climbing behaviour and an increase in immobility. This suggests that chronic pregnancy stress led to further alterations of the noradrenergic system that resulted in this differential behavioural outcome of imipramine administration. Future studies will therefore be necessary to examine whether the attenuated noradrenaline release within the PVN and the decreased receptor expression is prevented by pregnancy stress. In summary, interpreting depression-like behaviours in animal models of peripartum stress need to be judged with caution, as a

negative outcome might not necessarily indicate a non-depressive state in the dam. To that end, it would be interesting to use a different test to assess depression-like behaviour, such as the saccharin preference test, which would determine whether stressed dams display an anhedonic-like phenotype. Indeed, recent studies in stressed male mice have shown that while 10 days of social defeat reduced saccharine preference it did not alter FST or tail suspension test behaviour (Berton et al., 2006; Krishnan et al., 2008). Therefore, more studies should be employed before the effect of pregnancy stress on depression-like behaviour can categorically be stated.

In addition to the effect of chronic stress on depression-like behaviour, several studies revealed altered maternal behaviour after chronic pregnancy stress. Thus, both RS and ultra-mild stress during pregnancy results in decreased maternal aggression in the resident-intruder test, and an increased latency to retrieve the pups during the pup-retrieval test (Maestripieri et al., 1991; Pardon et al., 2000). However, basic maternal care was not changed as a result of chronic pregnancy stress (Pardon et al., 2000). Here, I could show that lactating dams that experienced chronic stress during pregnancy exhibited an increase in ABN (kyphotic nursing) from early- to mid-lactation compared with unstressed control dams. However, there are studies showing a decrease in ABN after repeated RS (Smith et al., 2004) or under chronic high levels of CORT (Brummelte and Galea, 2010a). The effects of acute separation from the pups, also a form of stress, also affects maternal behaviour with both a reduced maternal behaviour (Boccia et al., 2007) and a higher intensity of maternal care being observed (Francis et al., 1999). Interestingly, I revealed that repeated RS during lactation, which was coupled with pup separation, led to an increased display in maternal behaviour. In contrast, separation alone did not affect this behaviour. Thus, as has been

demonstrated in humans, chronic stress exposure during the peripartum period can lead to both increased and decreased maternal care / attachment (Barnett and Parker, 1986; Lyons-Ruth et al., 1986; Bifulco et al., 2004; Bridges, 2008).

Importantly, there appears to be a correlation between the effects of peripartum stress on maternal behaviour and the impact it has on anxiety-related behaviour. Thus, chronic pregnancy stress led to an increase in anxiety-related behaviour as assessed in the EPM. Chronic stress had no effect on anxiety-related behaviour in virgin animals, demonstrating the specificity of the chronic stress effect on this important peripartum adaptation. These results are in accordance with studies in rodents, non-human primates and humans showing that mothers with high levels of anxiety express a high maternal motivation and highly protective maternal mothering, known as “helicopter parenting” in humans (Maestripieri, 1993b, a; Bridges, 2008). In support, HAB dams show higher levels of ABN, leave the nest comparably less often and retrieve the pups faster during the pup retrieval test even under adverse and challenging conditions compared to LAB dams (Neumann et al., 2005a; Bosch, 2011). Given the observed changes in maternal behaviour and maternal anxiety after chronic pregnancy stress it might be of interest in human studies to assess patterns of maternal behaviour in the first week after birth to see if this factor might be an indicator for a subsequent onset of postpartum anxiety disorder.

Those behavioural changes might result, at least partly, from the stress effects on important peripartum-associated adaptations at the level of the hypothalamic PVN. Towards the end of pregnancy into lactation, there is typically an increase in the OXT system activity, which is reflected by an increased expression of hypothalamic OXT mRNA and its receptor (Zingg et

al., 1995; Windle et al., 1997; Bosch and Neumann, 2008; Figueira et al., 2008; Slattery and Neumann, 2008). Chronic pregnancy stress prevented the peripartum-induced rise in OXT mRNA within the PVN without altering AVP or CRH mRNA expression. This suggests that the decrease in OXT mRNA may be a critical result following pregnancy stress. Interestingly, a decreased OXT system activity might be linked to the elevated anxiety found after chronic pregnancy stress since OXT is known to act anxiolytic in lactating but not virgin or male rats (Neumann et al., 2000a). Admittedly, the reduction in OXT mRNA is in contrast to the observed changes in maternal care, as low OXT system activity is associated with low attachment rates and reduced maternal behaviour throughout species (Caldji et al., 1998; Francis et al., 2000; Maestripieri et al., 2009; Skrundz et al., 2011). However, for a more direct comparison with previous human studies I would have to assess plasma OXT levels. Moreover, it is known that OXT is rather required for the onset of maternal care, but not for its maintenance and that the anxiogenic effect of pregnancy stress might explain the pattern of maternal behaviour. Accordingly, high levels of anxiety are linked to increased levels of maternal behaviour in mice, rats and humans, suggesting that it is a biologically relevant, evolutionary adaptation (Maestripieri and D'Amato, 1991; Champagne and Meaney, 2006; Bosch and Neumann, 2008; Bridges, 2008; Braw et al., 2009; Hillerer et al., 2011a; Kessler et al., 2011).

2.3 Consequences of chronic stress on hippocampal neurogenesis

A more recently identified consequence of pregnancy and lactation is that dramatic alterations in hippocampal neurogenesis occur throughout this period (for review see

Pawluski et al., 2009). Moreover, neurogenesis is linked to stress-related diseases including depression and anxiety (Czeh et al., 2001; Santarelli et al., 2003; Revest et al., 2009; Dagyte et al., 2011; Snyder et al., 2011). Thus, both physiological and psychological stressors induce robust and reliable effects on cell proliferation and cell survival throughout different species, including mice, rats, tree shrews and monkeys (Uno et al., 1989; Gould et al., 1997; Gould et al., 1998; Tanapat et al., 2001; Falconer and Galea, 2003; Malberg and Duman, 2003; Pham et al., 2003; Torner et al., 2009), and loss of neurogenesis *via* knockout or x-ray irradiation leads to depression and exaggerated stress responses (Snyder et al., 2011). Further, OXT has recently been shown to promote neurogenesis (Leuner et al., 2010): Therefore, and given the known alterations in both the HPA axis and the OXT system during the peripartum period, I next wanted to determine whether stress during the peripartum period resulted in differential neurogenesis-related effects. However, before assessing the consequences of stress during the peripartum period, it was necessary to determine the effects of such stress exposure on male and virgin rats to reveal consequences that were specific to the peripartum period.

Therefore, in chapter 3, I investigated the effect of chronic RS on basal and stress-induced sex differences on adult hippocampal neurogenesis. In my studies, male and female rats were exposed to 2 h of RS for 12 consecutive days and were examined for basal and stress-induced CORT levels, neurogenesis parameters, including cell proliferation, cell survival, cell differentiation and stem cell quiescence, as well as spatial working memory. The choice of 12 days of stress rather than a longer period was to facilitate the comparison with stress during the lactation period, where it was important to ensure that the stress period remained firmly within the lactating period of rats.

Here, females exhibited higher basal CORT levels than males and these levels further rose across the chronic stress exposure timeframe, whereas stressed males were unaffected. Importantly, both males and females were shown to have a robust and reliable increase in plasma CORT during the restraint sessions. These results are in accordance with previous works in rats, demonstrating a different CORT response in adult males and females exposed to stress during adolescence, with only females responding with an increase in basal CORT levels after chronic RS (Barha et al., 2011). In addition, during stress exposure females showed higher peak CORT levels than males (Romeo et al., 2004) and returned faster to baseline levels (McCormick and Mathews, 2007). Given the observed basal and stress-induced differences in HPA axis reactivity in males and females, it would be of interest to assess adrenal parameters, like adrenal weight and sensitivity in future studies as I documented changes at this level following pregnancy stress, and others have reported similar observations in mice and rats (Reber et al., 2006; Reber and Neumann, 2008; Hillerer et al., 2011b). Assessing the HPA axis activity is of vital importance when interpreting the effects of chronic stress on adult hippocampal neurogenesis, as glucocorticoid levels are crucially involved in the regulation of hippocampal neurogenesis, especially after chronic stress exposure (Cameron and Gould, 1994; Cameron and McKay, 1999; Lemaire et al., 1999; Tanapat et al., 2001; Hellsten et al., 2002; Leuner et al., 2007).

As previously shown (Galea et al., 1997; Falconer and Galea, 2003; Westenbroek et al., 2004; Barha et al., 2011), I could prove that there are not only stress-induced, but also baseline sex differences in adult hippocampal neurogenesis. Accordingly, males expressed a higher number of immature neurons in the DG. Given the fact that the production of immature neurons seems to be functional relevant in hippocampal-dependent processes such as learning and memory (Gould et al., 1999; Shors et al., 2001) and sex differences in memory

tasks have already been described throughout species (de Frias et al., 2006; Shors, 2006a; Dalla et al., 2009; Dalla and Shors, 2009), I hypothesized that observed differences in immature neuron production would be reflected in a sex-dependent manner in spatial working memory. However, there was neither an effect of sex, nor of chronic stress exposure in a spatial working memory task. Admittedly, the specific functions contributed by adult-born neurons remain controversial and there is growing evidence that they might be rather involved in the acquisition of emotional relevant information (Hernandez-Rabaza et al., 2009) and the regulation of anxiety (Revest et al., 2009; Dagyte et al., 2011). Thus, simultaneous assessment of anxiety-related behaviour and hippocampal neurogenesis after chronic stress would greatly assist to reveal a potential link between differences in the number of immature neurons and this type of affective behaviour in males and females in future studies.

In accordance with previous work (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Pham et al., 2003; Heine et al., 2004), I revealed that chronic stress exposure attenuated cell proliferation in males, without effects on cell survival (Madeira et al., 1991; Madeira and Paula-Barbosa, 1993; Pham et al., 2003; Heine et al., 2004). Furthermore, the stress-induced decrease in cell proliferation was accompanied by alterations in stem cell quiescence. Interestingly, chronic stress led to a robust increase in stem cell quiescence with a concurrent decrease in actively cycling stem cells. This shift in stem cells from a proliferating status to quiescence can partly explain the reduced proliferation rate seen after chronic stress in males. Thus, increased stem cell quiescence might be a mechanism to reconstitute the niche after depletion of precursors (Kazanis et al., 2010; Knobloch and Jessberger, 2011) and might be important for maintenance / preservation of the stem cell pool under pathophysiological situations (Kandasamy et al., 2010). Although, cellular quiescence seems

to be actively maintained by distinct transcription programs (Coller et al., 2006), the exact regulatory mechanism of stem cell quiescence, particularly during chronic stress, are largely unknown. Therefore, future studies will focus on molecules that are involved in the regulation of stem cell quiescence, such as fibroblast growth factor (Palmer et al., 1995) under basal and chronic stress conditions.

In contrast to males, but accordant with previous work, exposure to chronic stress induced opposing effects in females, with no effect on cell proliferation (Westenbroek et al., 2004) or stem cell quiescence, but a robust decrease in the number of surviving cells (Westenbroek et al., 2004). Importantly, the analysis of differentiation patterns pointed out that the reduced cell survival in females must have been almost exclusively due to a death of neurons, as neuronal differentiation was reduced in contrast to an unchanged astroglial differentiation; an effect that was only seen in females but not in males. The reduced neuronal fate in females might be of clinical relevance, given the fact that women have an increased susceptibility to suffer from stress-related illnesses like major depression (Kendler, 1998; Shors and Leuner, 2003; Becker and Grilo, 2007), and that there seems to be a link between a reduced neurogenesis and depression (Czeh et al., 2001; Santarelli et al., 2003; Oomen et al., 2007; Snyder et al., 2011). Moreover, these results reveal that the elevation in basal CORT levels as seen in females affected solely neuronal survival without changing the number of proliferating cells, whereas proliferation in males seemed to be impaired by chronic stress exposure even though CORT levels were unchanged. Although, changes in stem cell quiescence after chronic RS might explain the decreased hippocampal cell proliferation in males, it does not justify the observed results in females, nor does it give information about a relationship between the observed changes in other neurogenesis parameters and CORT levels. However, it should be clearly kept in mind that there are other

factors beside CORT that could play a regulatory role. Based on a study in mice demonstrating that chronic i.p. administration of a CRH or AVP receptor antagonist reverses the stress-induced reduction in cell proliferation (Alonso et al., 2004) , it can be speculated that differences in CRH or AVP mRNA expression patterns could underlie the observed basal and stress induced sex differences in CORT levels and hippocampal neurogenesis. Therefore, future studies would be necessary to determine whether the stress protocol used in the present studies causes alterations in either neuropeptide. Furthermore, sex hormones might be involved in these differences given the fact that there is a known interaction of the hypothalamic-pituitary-gonadal (HPG) axis and the HPA axis (Kamel and Kubajak, 1987; Rabin et al., 1988), and the regulatory importance of gonadal hormones in adult hippocampal neurogenesis (for review see Galea et al., 2006). Interestingly, previous studies showed that oestrogen stimulates a transient increase in the number of newly generated cells in the DG, whereas it has no effect on longer survival times (for further details see below), which suggests that many new cells in the DG degenerate in females (Tanapat et al., 1999). Therefore, future studies will particularly assess oestrogen levels to draw a link between basal and stress-induced CORT levels and hippocampal neurogenesis.

Another possible explanation for the present findings is related to the trisynaptic circuitry of the hippocampus. The decrease in granule cell number following stress in females reported here in the DG could be due to anterograde and retrograde projections that occur between both the CA3 and CA1 regions of the hippocampus (Witter, 1989, 1993). As I focussed on the DG of the hippocampus, a conclusive statement about any such changes cannot be made. However, it is feasible that in females changes may have been first occurred in the CA3 region or other sub-regions of the hippocampus, with no change of cell proliferation in the DG, but in fact the changes in the other sub-regions may have contributed to the decrease in

the number of granule cells seen at the survival time-point. In line with this is the fact that only a small percentage of precursor cells in the DG express CORT receptors (Cameron et al., 1993a). Thus, it is possible that CORT may rather act indirect on neighbouring glial and neuronal cells that express appropriate receptors, which could then control the cell cycle of granule cells in the DG (Sousa and Almeida, 2002), *via* glutamate release in the DG (Stein-Behrens et al., 1994; Reagan and McEwen, 1997) or release of growth factors (Kuhn et al., 1997).

In summary, I demonstrated that males and females differ in basal and stress-induced CORT levels, as well as in different stages of adult hippocampal neurogenesis. Moreover, I thereby proved evidence that chronic RS induces robust effects in females, which was used as a basis for subsequent stress studies in females during the peripartum period.

Therefore, in chapter 4, I assessed the effect of chronic stress during lactation on hippocampal neurogenesis using the same paradigm as used in the male / female study discussed above. In the lactation paradigm, stress exposure started on LD2 for 12 days in order to ensure that the stress period remained firmly within the lactating period of rats.

As stated above, increased basal CORT levels are an important adaptation during the peripartum period. Moreover, the lactation-associated hypercorticism underlies the decrease in adult hippocampal neurogenesis observed during that time (Leuner et al., 2007). Thus, given the observed changes in CORT levels after chronic pregnancy stress it would be of interest to see whether the loss of the basal hypercorticism after pregnancy stress would consequently also lead to a reversal of the lactation-associated decrease in neurogenesis. However, such a study is infeasible due to the enormous hormonal fluctuations before, during and after birth, which would bias the stress effect on hippocampal neurogenesis

during lactation. Therefore, I studied the effects of chronic stress during lactation on adult hippocampal neurogenesis. I found a reduction in basal hippocampal cell proliferation and cell survival in lactating dams compared with virgins as previously shown by others (Darnaudery et al., 2007; Leuner et al., 2007; Pawluski and Galea, 2007). These findings gave me confidence in my paradigm and procedures, which enabled me to study more detailed aspects of hippocampal neurogenesis and the impact of stress on these adaptations.

Interestingly, I could show that the lactation-associated decrease in cell proliferation was prevented by chronic stress exposure, but actually led to a decrease in cell survival. These results might initially seem paradoxical, given the predominant assumption that chronic stress is thought to impair adult hippocampal neurogenesis (Gould et al., 1997; Czeh et al., 2002; Pham et al., 2003; Heine et al., 2005); an effect that is mainly believed to be due to increased basal CORT levels after chronic stress exposure (Gould et al., 1992; Cameron and Gould, 1994; Tanapat et al., 2001). Given the known effects of chronic pregnancy stress on basal CORT levels, it might be feasible that stress during lactation acts in a similar way and prevents the basal hypercorticism during lactation. However, stressed dams showed a robust activation of the HPA axis across the tested lactation period, with higher basal CORT levels observed on LD6 compared with non-stressed dams. Hence, it is likely that during chronic lactation stress there is a shift from CORT towards other regulatory mechanism playing a predominant role in adult hippocampal neurogenesis. One factor that might help to interpret the observed changes in cell proliferation and cell survival after chronic stress exposure is oestrogen, as it undergoes fluctuations across the peripartum period and has repeatedly been shown to affect hippocampal neurogenesis (Tanapat et al., 1999; Banasr et al., 2001; Ormerod et al., 2003; Tanapat et al., 2005). Oestrogen typically decreases in lactation from high levels in pregnancy (Smith and Neill, 1977) and is known to interact with

the HPA axis under conditions of stress (Handa et al., 1994). Importantly, it stimulates faster division of DG cells by regulation of the G_1/ S-phase transmission of the cell cycle (Geum et al., 1997; Hong et al., 1998). It may be hypothesized that chronic stress from the beginning of lactation might maintain high oestrogen levels in the dams, which, at least in part, would explain the observed changes in hippocampal cell proliferation and cell survival. Arguing for this theory are also the observed changes in the number of quiescent stem cells. While their number tended to be decreased as a consequence of lactation, chronic stress abolished this effect with a concurrent decrease in the number proliferating stem cells. Thus, chronic stress led to a decreased availability of actively cycling stem cells and, therefore, shortening the cell cycle length in the remaining available proliferating cells might be a compensatory mechanism after chronic stress exposure. However, the accelerated cell cycle might have detrimental effects in terms of the cell survival, as studies in hematopoietic stem cells revealed that increased levels of cell proliferation involve a higher frequency of replication errors (Arai et al., 2004; Hirao et al., 2004), which in fact would explain the reduced survival despite the increased proliferation in stressed dams. Beside this possible mechanism it has to be kept in mind that the generation and removal of cells is delicately balanced (Biebl et al., 2000; Kuhn et al., 2001) and incorporation of newborn cells above a critical set-point might also be maladaptive as observed following epileptic seizures (Varodayan et al., 2009). Interestingly, epileptic patients do not only express increased levels of cell proliferation due to changes in cell cycle length, but moreover an increased number of apoptotic cells. Consequently, increased apoptotic rates might also be a proposed mechanism in stressed dams to explain the results in cell proliferation and cell survival. The robust decrease in adult hippocampal neurogenesis seen during lactation and the prevention by chronic lactation stress raises the question about the functional importance of such lactation-associated

adaptations. It might be speculated that the decreased number of newly generated hippocampal cells is involved in the reduced stress response and anxiety during lactation (Neumann, 2001; Lonstein, 2007). Therefore, an increased number of proliferating cells as seen in lactation stressed dams might have long-term consequences on the HPA axis activity.

Beside stress induced changes in cell proliferation and cell survival, I showed that chronic stress led to a decrease in neuronal differentiation while astroglial differentiation was unchanged. These findings indicate that neurons were solely affected by the above described apoptotic processes in stressed dams, which is furthermore supported by the slight trend towards a decrease in the number of neuronal progenitor cells after lactation stress. The fact that solely neurons were affected by chronic stress exposure is an important finding, as the addition of neurons that reach maturity is thought to be of importance in relation to motherhood, especially maternal boding and care (Gandelmann et al., 1971; Santarelli et al., 2003; Leuner and Shors, 2006) and *vice versa* (Furuta and Bridges, 2009). Indeed, I could show a changed pattern of maternal care, i.e. an increase in ABN (chapter 5; and see below). This is an interesting finding as dams exposed to chronic stress during pregnancy showed an identical outcome in maternal behaviour (chapter 2) and were additionally shown to have a concurrent increase in anxiety. Although I did not assess hippocampal neurogenesis after chronic pregnancy stress for reasons mentioned above, it seems reliable that similar mechanisms are involved in the observed behavioural changes after chronic pregnancy or lactation stress. Hence, changes in neuronal differentiation patterns might underlie the alterations in anxiety and maternal behaviour seen after both stress paradigms. Although, there is no direct evidence for an involvement of newly generated neurons in anxiety-regulation, a recent study in mice showed that chronic mild

stress reduces the number of neural progenitor cells (DCX expressing cells), which is associated with an increase in anxiety- related behaviour (Dagyte et al., 2011).

Moreover, it has to be emphasized that a change in neuron numbers might not necessarily induce alterations in maternal care and anxiety *via* a direct pathway. As the hippocampus possesses direct and indirect connections to other brain regions, an indirect influence of hippocampal differentiation patterns on such behaviours would also be possible. In line with this, the hippocampus is connected to brain regions known for their importance in the regulation of anxiety and maternal behaviour, like the BNST, the amygdala and the PVN (Bosch et al., 2007a; Neumann, 2008; Bosch and Neumann, 2010). Thus, changes at the level of the hippocampus might indirectly contribute to alterations in those brain regions. Towards the end of pregnancy and into lactation, there is typically an increase in OXT system activity, which is reflected by an increased expression of hypothalamic OXT mRNA and its receptor (Zingg et al., 1995; Windle et al., 1997; Bosch and Neumann, 2008; Figueira et al., 2008; Slattery and Neumann, 2008). I demonstrated that chronic pregnancy stress prevented this peripartum-induced rise in OXT mRNA within the PVN. If the lactation stress paradigm results in a similar decrease in the activity of the OXT system, this could also explain the observed stress-reduction in neuronal differentiation.

The above mentioned link is suggested by a recent study of Leuner et al., which revealed an involvement of OXT in the regulation of adult hippocampal neurogenesis under basal and stress conditions. They could show that male rats that received repeated peripheral OXT injections expressed an increased amount of new neurons compared with vehicle treated animals. Interestingly, this effect was only observed in the ventral DG, but not the dorsal DG (Leuner et al., 2010). This is an important finding as the ventral hippocampus is connected with the amygdala, the medial prefrontal cortex and HPA axis structures (Sahay and Hen,

2007; Fanselow and Dong, 2010), and is thought to play a role in anxiety and stress regulation (Herman et al., 1995; Bannerman et al., 2004). Therefore, it would be of importance to determine the effect of the lactation-stress paradigm on the OXT system in future studies to determine whether it may be involved in the observed differences in hippocampal neurogenesis.

Given the results of my study demonstrating that chronic stress reduces cell survival and neuronal differentiation in lactating females, it would be of particular interest to determine neurogenesis in women suffering from PPD, to investigate if such changes might underlie the behavioural phenotype of this mental disorder.

In summary, the results of chapter 3 and 4 showed that chronic stress affects adult hippocampal neurogenesis in a sex-dependent manner, but moreover that it alters important peripartum adaptations at the level of the hippocampus. Thus, while chronic stress did not affect cell proliferation or stem cell quiescence in virgin animals, it reversed the lactation-associated reduction in proliferation and the increased efficiency in the stressed lactating group, illustrating the specificity and importance of such changes to the peripartum period. Interestingly, chronic stress reduced neuronal fate in virgin and lactating females, but not in males. Therefore, the observed alterations in neurogenesis might not only play a role in the behavioural and neuroendocrine adaptations observed following chronic lactation stress, but, moreover, might underlie the increased susceptibility for women to suffer from stress-related illnesses, including depression and anxiety disorders, particularly during the peripartum period.

3. Effects of early life stress

Beside the detrimental effects of chronic stress on numerous parameters in the dam, it is well established in rodents and humans that prenatal, but also postnatal stress has long-term consequences on the physiological and behavioural outcome of the offspring (Ward et al., 2000; Heim and Nemeroff, 2001; Weinstock, 2001; Meaney et al., 2002; Knackstedt et al., 2005; Mairesse et al., 2007; Darnaudery and Maccari, 2008; Heim et al., 2008; Miyagawa et al., 2011). Interestingly, chronic stress during pregnancy did not only cause an increase in anxiety-related behaviour in the dam, but, moreover, induced a similar phenotype in the adult offspring. Thus, prenatally stressed offspring showed alterations in the adult behaviour, as seen in an increase of anxiety-related and depression-like behaviour. Increased anxiety in adulthood seems to be a robust parameter after prenatal stress as it has been consistently shown in rats and mice (Fride and Weinstock, 1988; Ward et al., 2000; Nishio et al., 2001; Rice et al., 2007; Darnaudery and Maccari, 2008; Miyagawa et al., 2011) and might be due to the effects of stress on the developing CRH system of the amygdala (Cratty et al., 1995; Vallee et al., 1997; Dickerson et al., 2005; Estanislau and Morato, 2005; Zohar and Weinstock, 2011), which mediates the cognitive and physiological expression of fear and anxiety. In this line, Ward and colleagues showed that the increased adult anxiety after prenatal stress may underlie a change in CRH system activity, reflected by an increased CRH receptor expression in the amygdala of prenatal stressed adult males. Moreover, prenatally stressed rats showed an increase in defensive withdrawal after additional RS; an effect that could be blocked by prior icv infusion of a CRH antagonist (Ward et al., 2000). In addition to the prenatal stress effects on the CRH system, an impairment of the OXT system in the PVN has to be considered. The PVN develops from PD13 to PD15 onwards and is able to react on

maternal glucocorticoids from this stage onward (Fujioka et al., 1999; Fujioka et al., 2003). Therefore, it might be that prenatal stress affects the OXT system in the PVN, where it typically exerts and anxiolytic effect (Neumann, 2007; Blume et al., 2008). However, the underlying mechanisms for the changes seen at the molecular and behavioural level are not yet fully understood. Interestingly, there seems to be a link between the mothers' level of anxiety during pregnancy and the neurodevelopmental outcome of the child. Thus, a recent study in humans revealed that the prenatal trait anxiety before delivery is negatively correlated with the expression of placental 11β-HSD2 (O'Donnell et al., 2011). This barrier enzyme converts CORT to the inactive 11- dehydrocorticosterone (Brown et al., 1993; O'Donnell et al., 2009) and, thereby, prevents the majority of maternal CORT from crossing the placenta. Importantly, chronic RS in the last week of pregnancy has the same effect on 11β-HSD2 expression and enzyme activity in rats (Mairesse et al., 2007), as high prenatal anxiety in humans. A lower expression level of this enzyme consequently increases the amount of CORT reaching the foetus, which might affect above mentioned systems and thereby causes the observed behavioural alterations in the offspring. Arguing for the importance of the barrier enzyme is the fact that administration of the 11β-HSD2 inhibitor carbenoxolone results in an increased anxiety (Welberg et al., 2005), which mirrors the phenotype of the offspring observed after prenatal stress in my studies.

As already mentioned above, the anxiety-related phenotype of the prenatally stressed offspring was concurrent with an increase in depression-like behaviour. Given the known comorbidity between anxiety and depression, the results of the FST are in accordance with the outcome in anxiety. Moreover, they replicate the findings of previous studies demonstrating an increase in depression-like behaviour after prenatal stress exposure (Vallee et al., 1997; Smith et al., 2004).

While changes in adult mood and anxiety-related behaviours seem to be a common phenomenon after prenatal stress, postnatal stress appears rather to affect social components of behaviour, although changes in anxiety-related and depression-like behaviour have also been reported (Huot et al., 2001; MacQueen et al., 2003; Romeo et al., 2003; Aisa et al., 2008). However, in accordance with previous studies postnatal stress had no effect on anxiety (Caldji et al., 1998; Hulshof et al., 2011), while it led to a decrease in social behaviour. Changes in social behaviour after postnatal stress are a consistent finding throughout different species, including rats and monkeys (Dettling et al., 2002; Veenema and Neumann, 2009; Kleinhaus et al., 2010; Feng et al., 2011; Schulz et al., 2011) and might be due to alterations in neural circuits underlying social behaviour, such as the OXT and AVP system of the lateral septum and the piriform cortex. In line with this, MS decreases OXT-R expression in the lateral septum and increases V1a-R expression in the piriform cortex (Lukas et al., 2010a). Moreover, a blunted response of AVP has been shown to impair social recognition in adult MS rats (Lukas et al., 2010b). Consequently, a change in above mentioned systems might also explain the reduced social preference as seen in my study. Therefore, future studies will assess the effect of MS on brain regions undergoing particularly early postnatal plasticity and development, like the lateral septum, the hippocampus and the prefrontal cortex (Bremner et al., 1997; Huot et al., 2001; De Bellis et al., 2002).

One important factor that has to be considered when interpreting the adult outcome of the offspring is the quality of maternal care they receive during early life. The importance of maternal care in shaping the adult phenotype has been revealed by several cross-fostering studies in rodents (Francis et al., 1999; Priebe et al., 2005; Kessler et al., 2011) and nursery rearing in monkeys (Schneider et al., 2002). As mentioned in previous chapters, chronic

stress during both pregnancy and lactation led to an increase in active maternal care, as seen by augmented ABN levels. Interestingly, while the lower body weight gain appeared already after birth and persisted until adulthood in the prenatally stressed offspring, the effect of MS on body weight gain occurred primal in adulthood. These findings in body weight gain validate the efficacy of the two stress paradigms, and are a consistent finding throughout different experimental paradigms (Lesage et al., 2004; Buhl et al., 2007; Darnaudery and Maccari, 2008; Emack et al., 2008). *In utero*, the amount of CORT reaching the foetus might be quite high following gestational stress, whereas transmission of CORT *via* the milk is relatively low; regardless of the stress conditions. Furthermore, it has to be considered that postnatal stress occurs within the SHRP of the neonates, when the presence of the dam suppresses basal and stress-induced circulating CORT levels and the sensitivity of the pups to the stimulatory effect of stress is reduced (for review see Sapolsky and Meaney, 1986; Walker et al., 2001a). Consequently, disruption of mother-pup interaction in early life is likely to increase CORT in the pups, which might alter developing systems and thereby have long-lasting effects on the behavioural and physiological outcome in adulthood, but might not immediately have an effect on the physiological development of the offspring, which might moreover explain the later onset of body weight gain changes in the postnatally stressed *versus* prenatally stressed offspring. Although it seems that an increase in ABN might not be efficient to restore the harming effects of stress, it has to be considered that other factors like the amount of milk ejected per reflex or the milk content i.e. milk fat content could play an important role. Even though increased maternal behaviour appears to play a minor role on stress-induced physiological alterations, they are obviously of vital importance to restore at least some of the behavioural abnormalities after early life stress. Thus, as already mentioned before, MS alone led to a reduction in social preference, while

increased levels of ABN, as seen after additional stress of the dam, were able to restore the deficits in this behaviour. These results are in accordance with studies in primates showing that maternal care and mother-infant bonding are fundamental for the development of social skills, including social bonding (Suomi, 1997). Although there a previous studies in rats indicating that natural variations in maternal care may affect the outcome of fear and anxiety (Liu et al., 1997; Caldji et al., 1998; Caldji et al., 2000; Liu et al., 2000), my studies could not prove that increased maternal care restores the deficits in adult affective behaviour seen after prenatal stress. However, in order to state this conclusively, cross-fostering studies are required as an increased amount of nursing might have an effect on anxiety-related and depression-like behaviour as well. Given the observed findings, it seems that prenatal and postnatal stress induces long-lasting and robust changes in epigenetic programming that lead to changes in HPA axis activity and affective behaviour, as already shown before (Darnaudery and Maccari, 2008; Murgatroyd and Spengler, 2011). Moreover, natural variations in maternal behaviour have been shown to lead to altered epigenetic regulation of the glucocorticoid receptor (Weaver et al., 2004). Thus, the combination of stress and elevated nursing levels may interact to lead to differing epigenetic programming in the offspring. Finally, it has to be kept in mind that the restoring effect of maternal care might also be dependent on the type of behaviour that is affected after early life stress. Thus, it might be speculated that the early-life programming by prenatal- or postnatal stress which results in a change in adult affective behaviour, is an evolutionary adaptation to prepare the offspring for survival in a dangerous or impoverished environment, and that the increased maternal anxiety and care reflects the maternal perception of such danger (Glover, 2011).

4. Perspective for future studies

Although, the results of the present thesis provided important insights in the effects of chronic peripartum stress and early life stress, they also raise several questions providing a host of conceivable starting-points for future studies.

Given the observed attenuation of the postpartum-associated hypercorticism, together with the increase in adrenal weight, it would be of interest to examine whether changes at the level of the adrenal gland, i.e. adrenal insufficiency, mediate the observed decrease in basal CORT levels after pregnancy stress. Similarly, alterations in adrenal gland functioning might also apply to the observed basal and stress-induced sex differences in adult hippocampal neurogenesis. Analysis of adrenal sensitivity in males and females would provide more detailed information about HPA axis activity and the according patterns of neurogenesis in males and females.

However, as extensively discussed, CORT levels might not solely explain alterations in peripartum-associated adaptations. Thus, extending the studies to other hormonal parameters seems to be of vital importance for future studies. This particularly includes the analysis of CBG plasma levels, as these might influence the levels of free circulating CORT, as well as placental 11β-HSG2, given its importance in stress, maternal anxiety (O'Donnell et al., 2011) and the physiological development of the offspring (Mairesse et al., 2007). Moreover, examination of oestrogen and OXT levels might help to explain the observed changes in hippocampal neurogenesis in males, virgins and lactating females. With respect to the hypothesis that changes in cell cycle length might underlie the discrepancy in cell proliferation and cell survival in lactation stressed dams, the use of Ki-67 as a proliferation marker is a further option. In contrast to BrdU, which only labels cells in the S-phase, Ki-67

labels cells that are in either the G_1-, S-, G_2- or M-phase of the cell cycle and, thereby, provides more detailed information about the cell cycle length. Moreover, to further assess the possibility that the stress-induced decrease in cell survival seen in virgin and lactating females is solely due to a death of neurons performing a double-immunostaining with the neuronal marker NeuN and apoptotic markers such as caspase-3 could provide further insights. To expand the observed changes in maternal care following lactation stress, it would be interesting to assess anxiety and depression in this paradigm, which would also enable us to determine if a reciprocal relationship of these behaviours with alterations in hippocampal neurogenesis exists. Given the importance of the olfactory bulb in maternal behaviour (for review see Levy and Keller, 2009) and the fact that newly generated neurons from the SVZ migrate *via* the rostral migratory stream to the olfactory bulb, analysis of neurogenesis in the SVZ would expand our knowledge about the effects of chronic peripartum stress on adult neurogenesis and on maternal behavior.

The observed decrease in body weight gain after early life stress, despite the increase in active maternal care raises the question of whether the nutritional content might be changed due to an effect of peripartum stress. As the nutritional content seems to be an important factor in the regulation of the behaviour and the stress-response of the offspring (for review see Walker, 2010), analysis of milk content and the amount of milk ejected per reflex could provide further insight in the observed physiological and behavioural alterations seen after pre-or postnatal stress. Essential for future studies is also the analysis of neuronal systems that might be involved in the observed behavioural abnormalities seen after pre- and postnatal stress, particularly the CRH system of the amygdala, as well as the OXT and AVP system of the lateral septum and the PVN. Moreover, given the growing belief that epigenetic mechanisms might take place during early life stress (Darnaudery and Maccari,

2008; Murgatroyd and Spengler, 2011), it would be interesting to assess whether the different forms of early life stress lead to specific epigenetic markers that play a role in the behavioural profile observed in adulthood. Furthermore, maternal behaviour shows transgenerational similarities; determining whether the female offspring of pregnancy stressed dams shows a similar behavioural pattern in adulthood would be a nice addition to the already performed studies. Finally, as maternal care seems to play a crucial role in shaping the adult phenotype of the offspring, cross-fostering studies could provide further insight in the above discussed hypothesis.

5. Concluding remarks

Neuroendocrine, neuronal and behavioural peripartum adaptations are essential for the female`s successful adaptive response when becoming maternal. Despite these adjustive mechanisms, the period around birth represents a time with high risk for women to develop mood and anxiety disorders that include a severe confinement of mother-infant interactions. Unfortunately, the aetiology of postpartum mood and anxiety disorders is largely unknown. However, chronic stress during the peripartum period is one major risk factor of such mental illnesses. Therefore, my thesis centered on the premise that by assessing the consequences of peripartum stress, I can provide insight into the mechanisms underlying postpartum mood and anxiety disorders. In order to demonstrate the specificity of such effects to the peripartum period it is necessary to include controls such as males and, particularly, virgin females.

In this thesis, I demonstrated that chronic stress during either pregnancy or lactation prevented a number of the above mentioned peripartum adaptations.

Thus, I could show that chronic stress during pregnancy induces an increase in active maternal care with a concurrent increase in anxiety-related behaviour. Although, chronic stress and increased levels of anxiety are often associated with an increase in depression-like behaviour, I did not observe an effect of chronic stress in this direction. However, using the antidepressant imipramine, I revealed that chronic stress prevented the lactation-associated decrease in the noradrenergic system activity, which in general underlies the observed

passive stress-coping behaviour in the FST. Furthermore, I could show that chronic pregnancy stress prevented the lactation-associated basal hypercorticism and the elevation in the OXT system within the PVN. Illustrating the specificity of the stress-induced changes to the peripartum period, most of the parameters were not affected in stressed virgin females. Given the fact that several postpartum psychiatric disorders have been linked to changes in basal ACTH and cortisol levels in humans, the results of the present thesis provide further important evidence that HPA axis dysregulation may be one major mechanism involved in the development of postpartum mood and anxiety disorders.

Naturally, males and females differ in their sensitivity to stress and their stress responsiveness to several stressors. Using chronic restraint as the stress paradigm and adult hippocampal neurogenesis as the readout parameter, I could demonstrate that sex differences do not only occur in the basal HPA axis activity and pattern of hippocampal neurogenesis, but moreover that cell proliferation, cell survival, cell differentiation and stem cell quiescence are differentially affected by chronic stress in male and female rats. These results are of importance for various reasons. First, there is a correlation between chronic stress, neurogenesis and depression and second, females have twice a higher risk to develop depressive disorders compared to males. Thus, the results provide evidence that sex differences in hippocampal neurogenesis may underlie the increased depression-susceptibility in females. Taking these findings, I next assessed the effect of this RS paradigm on the altered neurogenesis that occurs in the peripartum period. Here, I revealed that chronic stress during lactation prevents lactation-associated changes in cell proliferation and survival, but moreover, I showed for the first time that differentiation patterns and stem cell quiescence are altered after chronic lactation stress. The lactation period is a time when the

bigger part of metabolic resources is needed to insure healthy survival of the offspring. Therefore, reducing growth processes like neurogenesis and simultaneously increasing efficiency might be an important adaptive mechanism of the lactation period.

Beside the effects of chronic stress on the mother, I provided a wide-ranging of basic knowledge about the effect of early life stress on the adult emotional and social behaviour in the offspring. Importantly, I demonstrated that not only the timing of the stressor plays a pivotal role, but moreover that postnatal maternal-infant interaction in the form of maternal care is seminal for the adult behavioural outcome of the offspring. While prenatal stress induced an anxiety-related and depression-like phenotype in adulthood, postnatal stress rather led to deficits in social behaviour. Although an increase in active maternal care was effective to restore the effect of postnatal stress on social behaviour, it did not restore prenatal induced stress effects. Thus, it seems that regulatory mechanisms that are fundamental for adult affective behaviour undergo a critical stage of development during the prenatal stage and a disruption of these processes might not be rescued by increased amounts of maternal care. However, a high quantity of maternal care might be able to restore deficits in social behaviour when occurring simultaneously with postnatal stress exposure. The neurobiological mechanisms underlying the observed time-dependent differences in behaviour have not been subject of investigation in this thesis. However, future studies will concentrate to identify mechanism that are involved in the regulation of adult affective and social behaviours that might also underlie psychopathologies like anxiety disorders, autism, attention deficit hyperactivity disorders and schizophrenia, which have all been associated with early life stress exposure.

Epilogue

"And remember: Postpartum depression is beyond your control. It is very real and it is more common that most people realize. You need to be a hero."

Brooke Shields "Down Came the Rain"

…however, being a hero might not be enough to overcome postpartum depression. Thus, the design of appropriate pharmaceuticals in conjunction with cognitive therapies seems to be of vital importance to treat this serious mental disorder.

With my studies, I tried to contribute to a better understanding of the aetiology of postpartum mood and anxiety disorders, by identifying systems that are altered by peripartum stress and may as such serve as possible therapeutic targets to treat postpartum mood and anxiety disorders. Given the fact that such disorders are typically characterised by altered mother-infant interactions that might increase the likelihood of adult psychopathologies in their children, better understanding and treatment options will be beneficial for both mothers suffering from postpartum mood disorders and their children.

Summary in German

Deutsche Zusammenfassung

In allen Säugetieren ist die Zeit der Schwangerschaft bzw. Trächtigkeit, Geburt und Laktation gekennzeichnet durch eine Vielzahl an physiologischen Veränderungen und Anpassungen, durch welche die werdende Mutter auf die neuen Aufgaben vorbereitet wird, welche die Laktation mit sich bringt. Dabei kommt es nicht nur zu Adaptationen in verschiedenen neuroendokrinen und neuronalen Systemen sondern, es werden auch verschiedene Verhaltensweisen an den neuen Abschnitt des weiblichen Lebens angepasst. Die auftretenden Veränderungen sind entscheidend für das Überleben des Nachwuchses. Es gibt jedoch immer mehr Hinweise darauf, dass die Anpassungen zudem von fundamentaler Bedeutung für die psychische Verfassung der Mutter in dieser Phase sind. Trotz dieser prä- und postnatalen Anpassungen besteht zu dieser Zeit ein erhöhtes Risiko der Mutter an mental-psychischen Störungen zu erkranken, wie beispielsweise Depression oder Angststörungen. Während bereits zahlreiche Risikofaktoren für postnatal-psychische Erkrankungen beschrieben wurden, sind die zu Grunde liegenden neurobiologischen Mechanismen weitgehend unbekannt. Neben einer medizinischen Vorgeschichte der Mutter für mental-psychische Störungen stellt chronischer Stress während der Schwangerschaft und Laktation einen der wichtigsten bekannten Risikofaktoren dar.

Das Ziel dieser Arbeit war es, den Einfluss von chronischem Stress während der peripartalen Periode auf neuroendokrine und neuronale Anpassungen, sowie auf mütterliches Verhalten und Emotionen wie Angst und Depression zu untersuchen und damit das Wissen über die zugrundeliegenden Mechanismen postnataler Depression und Angststörungen zu erweitern (Kapitel 2 und 4). Um dies zu erreichen, musste zunächst festgestellt werden, ob die

auftretenden Anpassungen spezifisch für die Zeit um die Geburt sind (Kapitel 2 und 3). Dies ist aus folgenden Gründen von besonderer Bedeutung: 1) Die während der peripartalen Periode auftretende Stress-Hyporesponsitivität könnte gegebenenfalls dazu führen, dass die im Versuch herbeigeführten, stress-induzierten Veränderungen nicht das selbe Ausmaß wie in virginen Tieren annehmen und 2) um einen genaueren Einblick in stress-assoziierte prä- und postnatale Störungen zu erhalten, deren Ausbruch charakteristischerweise mit der Schwangerschaft verbunden ist. Die unten aufgeführten Ergebnisse meiner Studien sollen daher einen wichtigen Beitrag zum besseren Verständnis der Ätiologie solch schwerwiegender psychischer Störungen leisten. Wie bereits erwähnt spielen die genannten Anpassungen während der peripartalen Periode zudem eine entscheidende Rolle für die physiologische und psychische Entwicklung der Nachkommen. Deshalb scheint es zudem von Bedeutung den Einfluss einer durch Stress induzierten Veränderung solcher Anpassungen auf die Nachkommen zu untersuchen, um Mechanismen zu identifizieren, welche physiologischen und psychischen Erkrankungen im Erwachsenenalter zu Grunde liegen könnten (Kapitel 5).

In Kapitel 2 meiner Arbeit habe ich untersucht, welche Auswirkung chronischer Stress während der Trächtigkeit auf die üblich auftretenden Anpassungen dieses besonders empfindlichen Zeitabschnitts hat. Um zu zeigen, dass auftretende, stress-induzierte Veränderungen spezifisch für die Zeit um die Geburt sind, wurden neben den trächtigen Tieren zusätzlich virgine Weibchen als Kontrolle mit einbezogen. Ich konnte dabei zeigen, dass chronischer Stress während der Trächtigkeit spezifisch eine ganze Reihe von Anpassungen verhindert, welche üblicherweise während der peripartalen Periode auftreten. So wurde nicht nur der bereits bekannte basale Hypercortisolismus und der Anstieg der OXT

mRNA Expression im PVN unterbunden, sondern zudem die laktations-induzierte Reduktion im Angstverhalten beeinträchtigt. Diese Änderung im Angstverhalten wurde darüber hinaus von einem Anstieg im mütterlichen Verhalten begleitet. Die ist besonders interessant, da anderslautende Ergebnisse sowohl aus der Grundlagenforschung als auch aus Beobachtungen am Menschen zeigen, dass eine erhöhte Ängstlichkeit mit einer abnormal erhöhten mütterlichen Fürsorge in Verbindung steht. Auch wenn eine direkte Verbindung zwischen dem Auftreten von Angststörungen und Depression bekannt ist, konnte ich keine Veränderungen bezüglich des depressions-ähnlichen Verhaltens nach chronischem Stress feststellen. Eine zusätzliche akute Behandlung der Tiere mit dem trizyklischen Antidepressivum Imipramin, welches in erster Linie auf das noradrenerge System wirkt, gab hier jedoch genaueren Aufschluss. Es zeigte sich, dass chronischer Stress während der Trächtigkeit die bekannte pharmakologische Wirkung des Antidepressivums in laktierenden Tieren umkehrt, was darauf schließen lässt, dass die mit der Laktation verbundene Dämpfung des noradrenergen Systems durch den chronischen Stress während der Schwangerschaft verhindert wurde.

In Kapitel 3 und 4 dieser Arbeit habe ich die Folgen von chronischem Stress auf die adulte Neurogenese im Hippocampus untersucht. Um Anpassungen der Neurogenese zu identifizieren, welche unmittelbar mit der peripartalen Periode in Zusammenhang stehen, war es zunächst von Bedeutung ein Stressmodell zu wählen das reproduzierbare Stresseffekte in Weibchen hervorruft. Da es natürliche, geschlechtsabhängige Unterschiede in der Stressanfälligkeit, sowie der Reaktion auf unterschiedliche Arten von Stress zwischen Männchen und Weibchen gibt, wurden zunächst Versuche in beiden Geschlechtern durchgeführt. Da bereits gezeigt werden konnte, dass chronischer Stress zu Veränderungen

der adulte Neurogenese im Hippocampus führt, habe ich in Kapitel 3 die Auswirkungen von chronischem Immobilisierungsstress auf diesen Parameter untersucht. Dabei konnte ich zeigen dass es sowohl basale als auch stressinduzierte Geschlechtsunterschiede in der adulten hippocampalen Neurogenese gibt. Da dies auch auf den Corticosteron-Spiegel im Plasma zutraf, ist es denkbar, dass dieser neuroendocrine Parameter zumindest teilweise den geschlechtsspezifischen Veränderungen im Hippocampus zu Grunde liegt. Im Einzelnen konnte ich nachweisen, dass chronischer Stress in männlichen Tieren zu einer Reduktion in der Zellproliferation führt, welche sich ebenfalls in einer Abnahme in proliferierenden Stammzellen und einer gleichzeitigen Zunahme „stiller“ (nicht aktiv proliferierender) Stammzellen wiederspiegelte. Während bei weiblichen Tieren die Zellproliferation von der Stress-Exposition unbeeinflusst schien, war hier eine starke Abnahme in der Anzahl an überlebenden Zellen zu beobachten. Auch wenn die neugebildeten Zellen in männlichen Tieren basal eine niedrigere Überlebensrate aufwiesen, so zeigte sich keine weiter Reduktion nach chronischem Stress. Ähnliche Geschlechtsunterschiede waren auch in Bezug auf die Anzahl neuronaler Vorläuferzellen zu beobachten, wobei hier die Anzahl in Männchen größer war als in Weibchen. Auch wenn solch neuronale Vorläuferzellen eine wichtige Rolle für das räumliche Gedächtnis zu spielen scheinen, war in diesem Fall kein Zusammenhang zu beobachten. Die Auswertung neuronaler und astroglialer Differenzierungsmuster zeigte, dass chronischer Stress spezifisch die Anzahl an neugebildeten Neuronen in weiblichen Tieren reduzierte. Neben der erwähnten Unterschiede in der adulten hippocampalen Neurogenese, waren ebensolche auch in den basalen und stressinduzierten Corticosteron-Spiegeln zu beobachten. Die Tatsache, dass es geschlechtsspezifische Unterschiede in der Aktivität der Stress-Achse (HPA -Achse) gibt, legt die Vermutung nahe, dass diese zumindest teilweise den Geschlechtsunterschieden in der Neurogenese zu Grunde liegen. Desweiteren

konnte mit diesen Versuchen gezeigt werden, dass chronischer Immobilisierungsstress ein gutes Modell darstellt Stresseffekte auf die hippocampale Neurogenese in Weibchen zu untersuchen.

In Kapitel 4 meiner Arbeit konnte ich nun mit dem durch Kapitel 3 erlangten Vorwissen untersuchen, welchen Einfluss chronischer Stress während der Laktation auf die adulte Neurogenese hat. Hier konnte ich die aus der Literatur bekannte Reduktion der Zellproliferation und Überlebensrate der Zellen während der Laktation bestätigen und damit die Integrität und Reproduzierbarkeit meines Stressmodells bekräftigen. Vor allem gelang es mir erstmals zu zeigen, dass chronischer Stress während der Laktation zu einer Veränderung im neuronalen/astroglialen Differenzierungsmuster, sowie der Anzahl „stiller“ Stammzellen führt. Die bereits erwähnte Reduktion in der Zellproliferation wurde durch den chronischen Stress aufgehoben, auch wenn die Anzahl an aktiv proliferierenden Stammzellen reduziert war. Ähnlich wie bereits in virginen Tieren beobachtet, war die Anzahl neuronaler Vorläuferzellen, sowie die astrogliale Differenzierung vom Stress unbeeinflusst, wohingegen die neuronale Differenzierung reduziert war. Die Auswertung des Corticosteron-Spiegels zeigte einen Anstieg nach chronischem Stress. Dementsprechend scheint es wahrscheinlich, dass neben der bekannten Wirkung von Corticosteron weitere Mechanismen an der Regulation der Neurogenese beteiligt sind. Die erhaltenen Daten scheinen von besonderem Interesse, da eine Anzahl von Studien bereits zeigen konnte, dass ein Zusammenhang zwischen hippocampaler Neurogenese und Depression besteht. So wäre es durchaus möglich, dass die beobachteten stress-induzierte Veränderungen in der Neurogenese dem erhöhten Depressionsrisiko der peripartalen Periode zu Grunde liegen.

Wie bereits erwähnt spielen Anpassungen die während der peripartalen Periode auftreten eine entscheidende Rolle für die physiologische und psychische Entwicklung der Nachkommen. Insbesondere die Beziehung zwischen Mutter und Kind kann die späteren Verhaltensmuster eines Kindes beeinflussen. Neben den Auswirkungen von chronischem Stress auf die Mutter und die damit einhergehenden Veränderungen im mütterlichen Verhalten, habe ich zudem untersucht, inwieweit sich pränataler oder postnataler Stress auf das adulte Verhalten des Nachwuchses auswirkt (Kapitel 5). Dabei was es mir möglich nachzuweisen, dass chronischer Stress in der frühen Lebensphase zu einer Reihe von Verhaltensauffälligkeiten im Erwachsenenalter führt, wobei der Zeitpunkt der Stressexposition, sowie die Quantität der mütterlichen Fürsorge eine entscheidende Rolle spielt. Während pränataler Stress (Immobilisierungsstress der Mutter) eher zu einer Zunahme affektiver Störungen führt, zeigen postnatal gestresste (maternal separierte) Nachkommen Defizite im Sozialverhalten. Interessanterweise, konnte ein Anstieg im aktiven mütterlichen Verhalten, wie nach chronischem Stress während der Schwangerschaft und Laktation beobachte, Defizite im Sozialverhalten reduzieren, wohingegen die Störungen im affektiven Verhalten des Nachwuchses davon unverändert blieben.

Zusammenfassend konnte ich in meiner Dissertation zeigen, dass chronischer Stress sich negativ auf eine Reihe von neuroendokrinen und molekularen Parametern, sowie auf bestimmte Verhaltensweisen auswirkt. Dabei war es mir nicht nur möglich zu zeigen, dass die Auswirkungen von chronischem Stress einem geschlechtsspezifischen Einfluss unterliegen, sondern dass sie zudem vom reproduktiven Status anhängig sind. Mit den

vorliegenden Studien kann ich zum Wissen über die weitreichenden Folgen von chronischem Stress während der peripartalen Periode, sowie von frühem Lebensstress in den Nachkommen einen wertvollen Beitrag leisten. Dies soll zu einem genaueren Verständnis der Ätiologie postnatal-psychischer Störungen beitragen, welches wiederum als solches das Auftreten von psychischen Störungen im Nachwuchs verhindern kann. So können die durch peripartalen Stress erzeugten Veränderungen in zahlreichen Systemen als Biomarker oder therapeutische Angriffspunkte für postnatale Angststörungen und Depression dienen und verdeutlichen zudem die Tragweite solcher Veränderungen in Bezug auf die Physiologie und das Verhalten der Nachkommen.

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Abbreviations

11β-HSD2	11beta-hydroxysteroid dehydrogenase type 2
ABN	arched back nursing
ACTH	adrenocorticotropic hormone
AUC	area under the curve
AVP	arginine-vasopressin
ANOVA	analysis of variance
BNST	bed nucleus of stria terminalis
BrdU	5-bromo-2’-deoxyuridine
CBG	corticosteroid-binding globulin
CORT	corticosterone
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
DAB	diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
EDTA	ethylenediamintetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus-maze
FSGB	fish skin gelatine buffer
FST	forced swim test
GCL	granule cell layer
GFAP	glial fibrillary acid protein

GR(s)	glucocorticoid receptor(s)
HAB	high anxiety behaviour
HPA axis	hypothalamic-pituitary-adrenal axis
HPG axis	hypothalamic-pituitary-gonadal axis
icv	intracerebroventricular
ip	intraperitonal
IMI	imipramine
LAB	low anxiety behaviour
LD	lactation day
LDB	light dark-box
LG	licking and grooming
MAM	methylazoxymethanol acetate
MDD	major depressive disorder
MR(s)	mineralocorticoid receptor(s)
mRNA	messenger ribonucleic acid
MS	maternal separation
MWU	mann whitney *u* test
NSC(s)	neuronal stem cell(s)
NeuN	neuronal nuclear antigen
NS	non-stressed
OC	overcrowding stress
OD	optical density

OXT	oxytocin
OXT-R(s)	oxytocin receptor(s)
PCNA	proliferating cell nuclear antigen
PD	pregnancy day
PND	postnatal day
PRL	prolactin
PRL-R(s)	prolactin receptor(s)
PVN	paraventricular nucleus
RS	restraint stress
S	stressed
SEM	standard error of the mean
SGZ	subgranular zone
SHRP	stress hyporesponsive period
SON	supraoptic nucleus
SOX2	sex determining region y-box 2
SPT	social preference test
SSC	saline-sodium citrate
SVZ	subventricular zone
TBS	tris-buffered saline
TuJ1	neuron-specific class III beta-tubulin
USV	ultrasonic vocalization
V1a-R	vasopressin 1a receptor

VEH vehicle

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CV and publications

Curriculum vitae

Dipl. Biol. Katharina Hillerer

Von-der-Tann-Str. 10, 93047 Regensburg

Personal details:

* January 1st, 1983 in Passau, Germany

Single, no children

1993 – 2002

Secondary School, Auersperg-Gymnasium, Passau, Germany

Scientific education:

2008 to data

PhD student in neurobiology in Prof. Dr. Inga D. Neumann`s laboratory at the University of Regensburg

2007

MSc (Diploma) student in biology in Prof. Dr. Inga D. Neumann`s laboratory at the University of Regensburg (Diploma/Master thesis: “Characterisation of chronic gestational stress on behavioural and molecular parameters in dams and their offspring”)

2005 – 2007

Graduate studies in biology at the University of Regensburg with focus on Zoology/Neurobiology (grade: 1.3), Cell biology (grade: 1.3) and Medical Microbiology (grade: 1.7)

2002 – 2005

Undergraduate studies at the University of Regensburg, Germany

Publications

Hillerer K.M., Reber S., Neumann I.D., Slattery D.A (2011) Exposure to chronic pregnancy stress reverses peripartum-associated adaptations: implications for postpartum mood and anxiety disorders. Endocrinology 2011 Oct;152(10):3930-40. doi: 10.1210/en.2011-1091

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