TITLE PAGE

**Lactation-induced reduction in hippocampal neurogenesis is reversed by repeated stress exposure**

Hillerer K.M.[1,2], Neumann I.D.[1], Couillard-Despres S.[4], Aigner L. [3,*], Slattery D.A[1]

[1]Department of Behavioural and Molecular Neurobiology, University of Regensburg, Germany

[2]Department of Obstetrics and Gynaecology, Salzburger Landeskrankenhaus (SALK), Paracelsus Medical University, Austria

[3] Institute of Molecular Regenerative Medicine, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Salzburg, Austria

[4]Institute of Experimental Neuroregeneration, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Salzburg, Austria

[*]**Corresponding author and reprint requests:** Ludwig Aigner, PhD, Institute of Molecular Regenerative Medicine, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Salzburg, Austria, Strubergasse 21, 5020 Salzburg, Austria. Tel: 0043-662-442002-1280, Email: ludwig.aigner@pmu.ac.at

**Running title:** Stress effects on hippocampal neurogenesis in lactating rats

**Keywords:** lactation, hippocampal volume, neurons, differentiation, corticosterone

**Disclosure:**

This manuscript is the peer-reviewed version of the article Hillerer KM, Neumann ID, Couillard-Despres S, Aigner L, Slattery DA (2014). Lactation-induced reduction in hippocampal neurogenesis is reversed by repeated stress exposure. Hippocampus 24(6):673-83. doi: 10.1002/hipo.22258. Epub 2014 Feb 19.

The final publication is available at: http://onlinelibrary.wiley.com/doi/10.1002/hipo.22258/epdf

ABSTRACT

The peripartum period is a time of high susceptibility for mood and anxiety disorders, some of them have recently been associated with alterations in hippocampal neurogenesis. Several factors including stress, ageing, and, perhaps unexpectedly, lactation have been shown to decrease hippocampal neurogenesis. Intriguingly, lactation is also a time of reduced stress responsivity suggesting that the effect of stress on neurogenic processes may differ during this period. Therefore, the aim of the present study was to assess the effect of repeated stress during lactation (2 h restraint stress from lactation day (LD) 2 to LD13) on brain weight, hippocampal volume, cell proliferation and survival, and on neuronal and astroglial differentiation. Besides confirming the known lactation-associated decrease in cell proliferation and survival, we could reveal that stress reversed the lactation-induced decrease in cell proliferation, while it did neither affect survival of newly born cells nor the number of mature neurons in lactation but did not alter immature neuron production or the number of astroglial cells. Stress exposure increased relative brain weight and hippocampal volume mirroring the observed changes in neurogenesis. Interestingly, hippocampal volume and relative brain weight were lower in lactation compared to nulliparous females under non-stressed conditions. This study assesses the effect of stress during lactation on hippocampal neurogenesis and indicates that stress 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 maternal behaviour, including maternal care and aggression (Hillerer et al., 2011a; Neumann, 2001; Russell et al., 2001; Walker et al., 1995). However, in addition to changes directly associated with reproductive functioning, a host of important behavioural and physiological alterations occur (Carter et al., 2001; Neumann, 2001; Neumann, 2003; Walker et al., 1995), with decreased anxiety (Altemus et al., 1995; Carter et al., 2001; Heinrichs et al., 2001) (and see (Slattery and Neumann, 2008) for review) and increased basal cortisol / corticosterone (CORT) levels amongst the most prominent. The latter alteration is related to the presence of nursing pups (Stern et al., 1973; Walker et al., 1995) and has been directly correlated with the concomitant decrease in hippocampal neurogenesis observed during the first postpartum weeks (Leuner et al., 2007).

In addition to the peripartum-associated hypercorticism / hypercortisolism, there are numerous alterations that act in concert to decrease the response of the HPA axis to external stressors (Altemus et al., 1995; Brunton et al., 2008; Heinrichs et al., 2001). This raises the intriguing possibility that stress during the peripartum period may affect neurogenesis in a different fashion to that observed in male and female (nulliparous) rodents. Thus, it has repeatedly been demonstrated that exposure to a variety of stressors decreases hippocampal cell proliferation and survival in males, and cell survival in females (Gould et al., 1997; Gould et al., 1998; Hillerer et al., 2013; Lucassen et al., 2001; Pham et al., 2003). Such stress-related alterations in neurogenesis have been posited to play a crucial role in the aetiology of anxiety and depression (Fuchs, 2007; Snyder et al., 2011). Moreover, there is a growing body of evidence suggesting that antidepressants modulate these stress effects, at least in part, by increasing hippocampal neurogenesis (Hanson et al., 2011; Snyder et al., 2011). In addition, numerous sex differences in various aspects of hippocampal neurogenesis, under both basal and stress conditions, have been reported (Galea et al., 2008; Hillerer et al., 2013). The peripartum period is a time of high susceptibility for mood and anxiety disorders (Beck, 2006; Bridges, 2008; Lonstein, 2007; Robertson et al., 2004) and stress during the peripartum period is one of the most prominent risk

factors to develop postpartum mood disorders (Robertson et al., 2004). Therefore, it may be hypothesized that alterations in hippocampal neurogenesis underlie some of the observed disturbances in postpartum mood. However, there is a lack of evidence for the impact of stress during lactation on postpartum hippocampal neurogenesis.

Therefore, the aim of our study was to determine the effects of repeated restraint stress (RS) during the lactation period on different aspects of hippocampal neurogenesis within the dentate gyrus (DG). We evaluated the number of proliferating and surviving cells, as well as neuronal and astroglial differentiation patterns of the proliferating cells. Moreover, as lactation has been shown to affect brain size in humans (Oatridge et al., 2002), we also evaluated absolute and relative brain weight, as well as hippocampal volume. Finally, we assessed the effect of stress on basal and acute-stress induced CORT levels across the stress period.

# MATERIALS AND METHODS

## *Animals*

Female Wistar rats (200-250g; Charles River, Sulzfeld, Germany), housed in groups of four in standard polycarbonate rat cages under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00h, 22°C ± 1°C, 60 ± 5% humidity; free access to food and water) and allowed to habituate for at least seven days were used in these studies. All experimental procedures were performed between 08:00 – 12:00, 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.

## *Experimental cohorts*

A total of three different experimental cohorts were used for the present studies. One cohort of animals (nulliparous n= 18; lactating n= 16) was used to analyze relative brain weigh, hippocampal volume hippocampal cell proliferation and immature neurons (see also *Figure 1A*), one cohort (nulliparous n= 15; lactating n= 14) was used to analyze hippocampal cell survival and cell fate, (see also *Figure 1B*) and a final cohort of animals (lactating non-stressed n= 13; lactating stressed n= 13) was used to assess plasma CORT levels.

## *Mating procedure and confirmation of pregnancy*

After habituation, all female rats were mated (two to three females to one male), and pregnancy was verified by vaginal smears (designated pregnancy day (PD)0). Non-pregnant rats were assumed to be nulliparous. All animals were returned to group cages with 4 females housed together (cage size 55 x 35 x 20 cm) until PD16 (or equivalent in nulliparous animals), when they were single-housed. ). To rule out the possibility of pseudopregnancy in nulliparous females and to confirm that these animals were normally cycling after mating, vaginal smears were taken on day 1 and day 3 after the end of the mating procedure. Females that were in proestrus on the day of perfusion were excluded from the study to rule out the possibility of estradiol-mediated changes in cell proliferation as shown before (Tanapat et al, 1999). On the day of birth, i.e. on 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*

Animals of the stressed groups were subjected to 2 h RS for 12 consecutive days (LD2-LD13, or equivalent in nulliparous animals) 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 (Hillerer et al., 2013; Luo et al., 2005; Pham et al., 2003; 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. Dams that were separated from their pups, but not exposed to stress, were also included to rule out an effect of pup separation on the parameters assessed. However, this group did not differ from non-stressed controls, which remained with their pups, in any parameter (data not shown). The body weight of each animal was recorded daily from the beginning of the stress procedure until the day of sacrifice.

*Nulliparous females*

In order to determine the effects of the reproductive status and stress on hippocampal neurogenesis in lactating females, the present study was carried out in parallel with our recent study (Hillerer et al., 2013). Therefore, the data on cell proliferation, cell survival and differentiation patterns from the nulliparous group is replicated here in order 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, brain weight and hippocampal volume in nulliparous and lactating females*

*BrdU labelling*

Proliferation: To examine proliferation of precursor cells, rats were injected with 5-bromo-2'-desoxyuridine (BrdU; 50mg/kg, i.p; 0.9% NaCl solution; Sigma/Aldrich.) once on the last day of

stress, immediately after removal from the restraint tubes, i.e 12:00. Twenty-four h after BrdU injection animals were deeply anesthetized with ketamine/ xylazine (90-120 mg/kg ketamine; 6-8mg /kg xylazin in 0.9% NaCl), intra-cardiacally perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. This time-point has been reported to be sufficient for newborn cells to complete one cell cycle (Takahashi et al., 1992).

Survival: To trace the survival and fate of recently born cells, rats received daily BrdU injections (50mg/kg, i.p.) during the first four days of stress exposure (LD2-LD5; or equivalent in nulliparous animals) as described above. Sixteen days after the last BrdU injection (LD21, or equivalent day in nulliparous animals) rats were anesthetized and perfused as described above. BrdU was freshly prepared in 0.9% NaCl solution to a dilution of 20mg/ml on each injection day (for a schematic representation of the study design see *Figure 1*)

*Histological procedures*

Immediately after 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)). Cresyl violet staining was performed on free-floating sections to analyze hippocampal volume. 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).

*Stereology*

To analyze hippocampal volume, pictures were taken at a 10x magnification and volume was assessed using polygon selection measurement in ImageJ. 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 100x 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.

*Experiment 3: Effect of RS exposure on plasma CORT levels*

As CORT levels have been shown to be an important regulator of neurogenesis (Barha et al., 2011; Galea et al., 2008; Magarinos and McEwen, 1995), we measured plasma CORT levels under basal and stress conditions. Therefore, a jugular vein surgery was performed on PD19 - as previously described (Hillerer et al., 2013; Neumann et al., 1998). 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 (30,000 IU/ml; Centravet, Bad Bentheim, Germany). Five days after surgery, on the first day of stress procedure (LD2), and on 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. Immediately after the basal sampling, rats were placed into the restraint tubes and additional blood samples were taken 30 and 90 minutes after start of the stress procedure. After 2h, rats were removed from the restraint tubes and returned to 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 (DRG Instruments GmbH, Marburg, Germany).

*Statistical analysis*

All data are expressed as mean ± SEM and were statistically 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. ANOVAs were calculated separately for number of cells in the dentate gyrus with group (nulliparous/lactating and control/stress) as between-subject factors. 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); overall statistics are shown in Table 1. Due to technical issues some sections could not be analyzed (proliferation and immature neurons: one nulliparous non-stressed animal, one lactating non-stressed and three lactating stressed animals; survival: two lactating stressed animals; neuronal/astroglial differentiation: two nulliparous non-stressed animals).

## RESULTS

*Stress protects from lactation-associated hippocampus volume shrinkage (Experiment 1)*

To assess the effects of the reproductive status and stress on relative brain weight and on hippocampal volume animals were sacrificed after 12 days of RS on LD13 (or equivalent in nulliparous animals; *see Figure 1A)*. Lactating animals showed decreased absolute (data not shown) and relative brain weight (Fig. 2A) under both basal and RS conditions when compared with nulliparous animals (Fig. 2A; $p<0.01$). However, RS animals, either nulliparous or lactating, showed an increase in relative brain weight compared with non-stressed rats (Fig. 2A; $p<0.05$). An even more pronounced effect was present at the level of the hippocampus volume. Compared to nulliparous animals, lactating animals showed a decrease in hippocampal volume under basal conditions (Fig. 2B, 2C; $p<0.001$). Whereas repeated stress led to a decrease in hippocampal volume in nulliparous animals (Fig. 2B; $p<0.01$), it led to an increase in the lactating group (Fig. 2B; $p<0.001$), resulting in comparable hippocampus volumes between nulliparous non-stressed animals and lactating stressed dams. Figure 2C shows a representative image of a cresyl violet staining (lactating stressed animal (a) and lactating non-stressed animal (b).

*Repeated RS exposure elevates cell proliferation in the DG SGZ during lactation, but does not affect the number of doublecortin expressing cells (Experiment 1)*

To assess the effects of the reproductive status and stress on cell proliferation animals were given a single BrdU injection after 12 days of RS (LD13 or equivalent in nulliparous animals) and were perfused 24 h later (see *Figure 1A*). Lactating animals showed a decreased number of BrdU-positive cells both under basal and repeated stress conditions (Fig. 3A; $p<0.05$). However, stressed lactating animals showed an increased proliferation rate compared to non-stressed dams (Fig. 3A; $p<0.01$).

Next we questioned if the stress-associated effects on hippocampal cell proliferation in lactating animals translated into different numbers of immature neurons by assessing the number of DCX positive cells in the DG. The analysis revealed no significant differences in the number of DCX positive cells (Fig. 3B). However, the morphology of DCX cells in the stressed group appeared to be less mature compared to non-stressed animals, as indicated by a less elaborated dendrite arborisation.

Figure 3C shows a representative image of a DAB-immunostaining (lactating non-stressed animal (I) and lactating stressed animal (II)) against DCX.

*Reproductive status and repeated RS modulate the fate of newly born cells in the DG (Experiment 2)*

We next assessed the effect of reproductive status and stress on the fate of newly born cells in the DG. Therefore, animals received BrdU injections on the first 4 days of stress (LD2-LD5 or equivalent in nulliparous and non-stressed groups) and were perfused 16 days later on LD21 (or equivalent in nulliparous animals; see *Figure 1B*). The quantitative analysis revealed a reduction in the number of BrdU-positive cells in non-stressed and stressed lactating animals and in stressed nulliparous animals compared to the non-stressed nulliparous animals (Fig. 4A; $p<0.01$) (Fig. 4A; $p>0.05$).

To evaluate if nulliparous and lactating animals vary in their astroglial and neuronal differentiation patterns under basal and RS conditions the hippocampus was analyzed for cells double-positive for BrdU and NeuN for neuronal differentiation, or BrdU and GFAP for astroglial differentiation. Although there was no effect of reproductive status on astroglial or neuronal differentiation, nulliparous and lactating rats that were exposed to RS showed a reduction in neuronal differentiation (Fig. 4B; $p<0.05$ versus respective non-stress groups), astroglial differentiation patterns were unchanged by RS exposure (Fig. 4C). Figures 4D-F shows representative images of BrdU/NeuN/GFAP triple labeling of the DG (4D: BrdU; 4E: NeuN; 4F: GFAP; 4G: merge).

*Repeated RS leads to an increase in basal CORT levels on LD6 (Experiment 3)*

To assess the effect of stress during lactation on CORT levels, basal blood samples were taken immediately prior to stress exposure on LD2, LD4 and LD6 in separate groups of lactating rats. 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 revealed higher basal CORT levels in RS dams on LD6 compared with non-stressed dams ($p<0.01$; Fig. 5). Acute stress-induced CORT levels did not significantly differ in between groups (data not shown).

## DISCUSSION

This study provides the first evidence that hippocampal morphology and distinct stages of adult hippocampal neurogenesis are affected by stress in lactation. Lactating rats showed a decrease in absolute and relative brain weight during mid-lactation, which reflects previously published MR studies in humans revealing reduced brain size during the peripartum period (Oatridge et al., 2002). The observed lactation-associated effect on brain weight was reversed repeated stress exposure. Changes in brain weight were also reflected in hippocampal volume and hippocampal cell proliferation. As previously reported, lactating dams showed a decrease in cell proliferation in the DG of the hippocampus compared with nulliparous females (Darnaudery et al., 2007; Leuner et al., 2007), which was reversed by stress exposure. Stressed dams displayed a lower number of dividing cells during the first days of lactation and survived for approximately two weeks, as well as a reduced neuronal differentiation, whereas immature neuron production and astroglial differentiation were unaffected by RS in lactation. 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, such as prolactin (Torner et al., 2009). Collectively, these findings highlight that repeated stress during lactation has detrimental effects on physiologically occurring postpartum-associated adaptations at the level of the hippocampus.

*Repeated RS during lactation prevents the lactation-associated decrease in hippocampal volume and cell proliferation and leads to a decrease in cell survival*

The brain, and more specifically hippocampal morphology, undergo a postpartum reorganization (Galea et al., 2000; Oatridge et al., 2002), which includes dendritic pruning of CA3 and CA1 pyramidal neurons of the hippocampus at the time of weaning, as well as an increase in spine density in these regions during pregnancy and lactation (Pawluski and Galea, 2006; Kinsley et al., 2006). Interestingly, we were able to show a reduction in hippocampal volume during lactation, which mirrors the decrease in hippocampal cell proliferation and cell survival seen in our study; as well as in other previous findings (Darnaudery et al., 2007; Leuner et al., 2007; Pawluski and Galea, 2007). The

above mentioned changes in hippocampal neurogenesis have been shown to be related to the lactation period and are associated with the hypercorticism observed during that time (Leuner et al., 2007). Interestingly, we could show that the lactation-associated decrease in hippocampal volume and cell proliferation was prevented by stress exposure. Despite these reversals, repeated RS actually led to a decrease in cell survival, as well as an increase in basal CORT levels one day after the last BrdU injection. These results may initially seem paradoxical, as psychosocial and physical stress are commonly thought to impair adult hippocampal neurogenesis (Czeh et al., 2002; Gould et al., 1997; Heine et al., 2005; Pham et al., 2003), mainly due to an effect of increased basal CORT levels (Cameron and Gould, 1994; Gould et al., 1992; Tanapat et al., 2001). However, these studies have been performed in males, whereas the general consensus of studies in females is that stress reduces cell survival (Hillerer et al., 2013; Kuipers et al., 2006) either without affecting the number of proliferating cells (Falconer and Galea, 2003; Hillerer et al., 2013; Westenbroek et al., 2004), or increasing the number of proliferating cells in virgin or pregnant females (Pawluski et al., 2011).Thus, it appears that males and females react differentially to elevated CORT levels and stress situations in general. Additionally, it has been shown that the DG proliferation rate may habituate to stress exposure and, thus, 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 stress and lactation, only a small percentage of precursor cells express CORT receptors (Cameron et al., 1993a). In addition, a decrease in hippocamapal [$^{3}$H]-Dexamethasone binding capacity (Meaney et al., 1989), as well as reduced coriticosteroid binding globulin levels (Pawluski et al., 2009), during lactation might play a regulatory role in this context. Another consideration is that there might be a shift from CORT towards other regulatory mechanisms playing a predominant role during lactation stress. Given the fact that repeated stress during lactation led to an increase in active (i.g. arched back nursing) behaviour (data not shown) in the 2h after stress and pup exposure has been shown to increase hippocampal cell proliferation in nulliparous rats (Pawluski and Galea 2007) it is possible that maternal behaviour itself might regulate hippocampal cell proliferation in stressed dams. 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). It is tempting to speculate that a similar situation may apply to the lactation period, as the normal decrease in cell proliferation may be involved in the reduced acute stress responsiveness observed at this time (Lonstein, 2007; Neumann, 2001). Therefore, increased cell proliferation as seen in stressed lactating animals may have long-term consequences on HPA axis activity.

In addition, closer inspection of hippocampal cell proliferation and survival in both lactation groups revealed that a higher percentage of cells died in stressed dams over the distinct time course assessed compared with non-stressed dams. This suggests that the lactation period, under normal circumstances, is associated with a greater efficiency in neurogenic processes, as fewer proliferating cells are required to result in the same net cell survival as occurs in nulliparous females / males, which is in accordance with previous studies (Cameron et al., 1993b; Tanapat et al., 2001). In contrast, increased cell death occurred in nulliparous animals 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 activity 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 survival in the stressed dams could indicate increased apoptosis of newly generated cells and, thereby, suggest a compensatory increase in cell proliferation as a result of an increased cell death of older cells after stress exposure (Abrous et al., 2005; Gould et al., 1991; Lucassen et al., 2001).

Another possible explanation for the differences observed between stressed and non-stressed dams is an alteration in cell cycle length, as speculated above. Interestingly, increased cell proliferation in epilepsy is not only associated with an increase in 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, as it decreases in lactation from high levels throughout 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, repeated stress may maintain high oestrogen levels in the dams, which, at least in part, would explain the observed changes in hippocampal cell proliferation and 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 gain better insight in factors regulating cell proliferation and survival under stress conditions during lactation.

*Repeated RS during lactation does not affect immature neuron production, but 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 neurogenic components (Lledo et al., 2006). Disruption of these processes by environmental changes or stress exposure may cause a lack of cell populations with specific, temporally-necessary, properties in hippocampal function (Abrous et al., 2005). Although immature neuron production during lactation was found to be decreased in a previous study (Leuner et al., 2007), we could not replicate this in our study. However, the discrepancy may be explained by the use of different immature neuronal lineage markers (TuJ1 in the former and DCX in ours). In agreement with previous findings in male and nulliparous rats (Hillerer et al., 2013; Pham et al., 2003), stress during lactation did not affect neuronal progenitor cells. Again, these results may initially appear to differ from the cell proliferation and survival results. However, upon closer inspection, despite not affecting the number of immature neurons, repeated stress appears to affect dendritic morphology of immature neurons; particularly in stressed dams (see Figure 3C/I and II). In more detail, we observed distraction of dendritic processes, including a lack of orientation towards the hilus of the DG. This is an interesting observation, as dendritic morphology and spine density has already been shown to be increased during the peripartum period in the prefrontal cortex and hippocampus (Kinsley et al., 2006; Leuner and Gould, 2010) and given the fact that immature neurons play an important role as pattern integrators and pattern separation (for review see (Aimone et al., 2010 #70)). Therefore, future studies should include a more detailed analysis of immature neurons in the DG of the hippocampus. Aside from the observed changes in dendritic morphology of immature neurons, we also observed a trend towards a decrease in immature neuron production, which might facilitate the

interpretation of our results. With respect to the hypothesized stress-induced increase in apoptosis, it seems feasible that the unchanged immature neuron production in stressed dams mirrors the shift from the increased proliferation towards decreased survival, as also occurred in nulliparous females. This becomes even more evident when considering the neuronal and astroglial differentiation patterns. Although neuronal differentiation was unchanged during the lactation period, stress exposure led to a reduction in mature neurons with a concurrent trend towards an increase in mature astroglial cells. These results reveal that DG 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 in the context of maternal bonding and care (Gandelmann et al., 1971; Leuner and Shors, 2006; Santarelli et al., 2003) and *vice versa* (Furuta and Bridges, 2009). Therefore, alterations in the number of neurons within the hippocampus might both directly and indirectly *via* DG pathways to other brain regions involved in maternal behaviour contribute to changes in maternal care and maternal anxiety, as seen after stress exposure during the peripartum period (Hillerer et al., 2011a; Maestripieri and D'Amato, 1991; Purcell et al., 2011).

## CONCLUSIONS

The results of the present study reveal important consequences of repeated stress exposure during lactation on maternal brain adaptations and particularly on different stages of adult hippocampal neurogenesis in the DG. The observed changes suggest that stress prevents or reverses the physiologically reduced proliferation and increased efficiency of neurogenesis in the peripartum period. These stress-induced changes in neurogenesis are likely to be mediated, at least in part, *via* increased apoptosis or a shortened cell cycle length, given the reduced neuronal differentiation observed in the stressed group. They may further play a role in the behavioural and neuroendocrine adaptations observed following lactation stress exposure. However, further research is required to elucidate specific regulatory mechanisms of cell proliferation, selection, differentiation and integration of newly generated cells which are altered by chronic stress. This study reveals that repeated postpartum stress exerts a variety of negative effects on the maternal brain and particularly in specific stages of hippocampal neurogenesis that might underlie the increased risk for depressive disorders during that susceptible time.

## ACKNOWLEDGMENTS

We thank Dr. Mahesh Kandasamy for support of staining procedures, and Rodrigue Maloumby, Andrea Havasi and Gabriele Schindler (University of Regensburg) for technical assistance. Moreover we would like to thank the following funding agencies for their support: the Deutsche Forschungsgemeinschaft (DFG SL141/4-1; D.A.S/ I.D.N.), the Bavarian State Ministry of Sciences, Research and the Arts (ForNeuroCell grant) (L.A.), the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreements n° HEALTH-F2-2011-278850 (INMiND, LA) and HEALTH-F2-2011-279288 (IDEA, LA), by the FWF Special Research Program (SFB) F44 “Cell Signaling in Chronic CNS Disorders” (LA), and by the state of Salzburg (to L.A.).

REFERENCES

Abrous DN, Koehl M, Le Moal M. 2005. Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85(2):523-69.

Aimone JB, Deng W, Gage FH. 2010. Adult neurogenesis: integrating theories and separating functions. Trends Cogn Sci 14(7):325-37.

Altemus M, Deuster PA, Galliven E, Carter CS, Gold PW. 1995. Suppression of hypothalmic-pituitary-adrenal axis responses to stress in lactating women. J Clin Endocrinol Metab 80(10):2954-9.

Barha CK, Brummelte S, Lieblich SE, Galea LA. 2011. Chronic restraint stress in adolescence differentially influences hypothalamic-pituitary-adrenal axis function and adult hippocampal neurogenesis in male and female rats. Hippocampus.

Beck CT. 2006. Postpartum depression: it isn't just the blues. Am J Nurs 106(5):40-50; quiz 50-1.

Bridges. 2008. Neurobiology of the parental brain.

Brunton PJ, Russell JA, Douglas AJ. 2008. Adaptive responses of the maternal hypothalamic-pituitary-adrenal axis during pregnancy and lactation. J Neuroendocrinol 20(6):764-76.

Cameron HA, Gould E. 1994. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61(2):203-9.

Cameron HA, Woolley CS, Gould E. 1993a. Adrenal steroid receptor immunoreactivity in cells born in the adult rat dentate gyrus. Brain Res 611(2):342-6.

Cameron HA, Woolley CS, McEwen BS, Gould E. 1993b. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56(2):337-44.

Carter CS, Altemus M, Chrousos GP. 2001. Neuroendocrine and emotional changes in the post-partum period. Prog Brain Res 133:241-9.

Czeh B, Welt T, Fischer AK, Erhardt A, Schmitt W, Muller MB, Toschi N, Fuchs E, Keck ME. 2002. Chronic psychosocial stress and concomitant repetitive transcranial magnetic stimulation: effects on stress hormone levels and adult hippocampal neurogenesis. Biol Psychiatry 52(11):1057-65.

Darnaudery M, Dutriez I, Viltart O, Morley-Fletcher S, Maccari S. 2004. Stress during gestation induces lasting effects on emotional reactivity of the dam rat. Behav Brain Res 153(1):211-6.

Darnaudery M, Perez-Martin M, Del Favero F, Gomez-Roldan C, Garcia-Segura LM, Maccari S. 2007. Early motherhood in rats is associated with a modification of hippocampal function. Psychoneuroendocrinology 32(7):803-12.

Falconer EM, Galea LA. 2003. Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. Brain Res 975(1-2):22-36.

Fuchs E. 2007. Neurogenesis in the adult brain: is there an association with mental disorders? Eur Arch Psychiatry Clin Neurosci 257(5):247-9.

Furuta M, Bridges RS. 2009. Effects of maternal behavior induction and pup exposure on neurogenesis in adult, virgin female rats. Brain Res Bull 80(6):408-13.

Galea LA, Ormerod BK, Sampath S, Kostaras X, Wilkie DM, Phelps MT. 2000. Spatial working memory and hippocampal size across pregnancy in rats. Horm Behav 37(1):86-95.

Galea LA, Uban KA, Epp JR, Brummelte S, Barha CK, Wilson WL, Lieblich SE, Pawluski JL. 2008. Endocrine regulation of cognition and neuroplasticity: our pursuit to unveil the complex interaction between hormones, the brain, and behaviour. Can J Exp Psychol 62(4):247-60.

Gandelmann R, Zarrow MX, Denenberg VH, Myers M. 1971. Olfactory bulb removal eliminates behavior in the mouse. Science 171 (967):210-211.

Geum D, Sun W, Paik SK, Lee CC, Kim K. 1997. Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. Mol Reprod Dev 46(4):450-8.

Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS. 1992. Adrenal hormones suppress cell division in the adult rat dentate gyrus. J Neurosci 12(9):3642-50.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E. 1997. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17(7):2492-8.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E. 1998. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 95(6):3168-71.

Gould E, Woolley CS, Cameron HA, Daniels DC, McEwen BS. 1991. Adrenal steroids regulate postnatal development of the rat dentate gyrus: II. Effects of glucocorticoids and mineralocorticoids on cell birth. J Comp Neurol 313(3):486-93.

Hanson ND, Owens MJ, Nemeroff CB. 2011. Depression, Antidepressants, and Neurogenesis: A Critical Reappraisal. Neuropsychopharmacology.

Heine VM, Zareno J, Maslam S, Joels M, Lucassen PJ. 2005. Chronic stress in the adult dentate gyrus reduces cell proliferation near the vasculature and VEGF and Flk-1 protein expression. Eur J Neurosci 21(5):1304-14.

Heinrichs M, Meinlschmidt G, Neumann I, Wagner S, Kirschbaum C, Ehlert U, Hellhammer DH. 2001. Effects of suckling on hypothalamic-pituitary-adrenal axis responses to psychosocial stress in postpartum lactating women. J Clin Endocrinol Metab 86(10):4798-804.

Hillerer KM, Neumann ID, Couillard-Despres S, Aigner L, Slattery DA. 2013. Sex-dependent regulation of hippocampal neurogenesis under basal and chronic stress conditions in rats. Hippocampus.

Hillerer KM, Neumann ID, Slattery DA. 2011a. From Stress to Postpartum Mood and Anxiety Disorders: How Chronic Peripartum Stress Can Impair Maternal Adaptations. Neuroendocrinology.

Hillerer KM, Reber SO, Neumann ID, Slattery DA. 2011b. Exposure to chronic pregnancy stress reverses peripartum-associated adaptations: implications for postpartum anxiety and mood disorders. Endocrinology 152(10):3930-40.

Hong J, Shah NN, Thomas TJ, Gallo MA, Yurkow EJ, Thomas T. 1998. Differential effects of estradiol and its analogs on cyclin D1 and CDK4 expression in estrogen receptor positive MCF-7 and estrogen receptor-transfected MCF-10AEwt5 cells. Oncol Rep 5(5):1025-33.

Kandasamy M, Couillard-Despres S, Raber KA, Stephan M, Lehner B, Winner B, Kohl Z, Rivera FJ, Nguyen HP, Riess O and others. 2010. Stem cell quiescence in the hippocampal neurogenic niche is associated with elevated transforming growth factor-beta signaling in an animal model of Huntington disease. J Neuropathol Exp Neurol 69(7):717-28.

Kinsley CH, Trainer R, Stafisso-Sandoz G, Quadros P, Marcus LK, Hearon C, Meyer EA, Hester N, Morgan M, Kozub FJ and others. 2006. Motherhood and the hormones of pregnancy modify concentrations of hippocampal neuronal dendritic spines. Horm Behav 49(2):131-42.

Kuipers SD, Trentani A, Westenbroek C, Bramham CR, Korf J, Kema IP, Ter Horst GJ, Den Boer JA. 2006. Unique patterns of FOS, phospho-CREB and BrdU immunoreactivity in the female rat brain following chronic stress and citalopram treatment. Neuropharmacology 50(4):428-40.

Leuner B, Gould E. 2010. Dendritic growth in medial prefrontal cortex and cognitive flexibility are enhanced during the postpartum period. J Neurosci 30(40):13499-503.

Leuner B, Mirescu C, Noiman L, Gould E. 2007. Maternal experience inhibits the production of immature neurons in the hippocampus during the postpartum period through elevations in adrenal steroids. Hippocampus 17(6):434-42.

Leuner B, Shors TJ. 2006. Learning during motherhood: A resistance to stress. Horm Behav 50(1):38-51.

Lledo PM, Alonso M, Grubb MS. 2006. Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7(3):179-93.

Lonstein JS. 2007. Regulation of anxiety during the postpartum period. Front Neuroendocrinol 28(2-3):115-41.

Lucassen PJ, Vollmann-Honsdorf GK, Gleisberg M, Czeh B, De Kloet ER, Fuchs E. 2001. Chronic psychosocial stress differentially affects apoptosis in hippocampal subregions and cortex of the adult tree shrew. Eur J Neurosci 14(1):161-6.

Luo C, Xu H, Li XM. 2005. Quetiapine reverses the suppression of hippocampal neurogenesis caused by repeated restraint stress. Brain Res 1063(1):32-9.

Maestripieri D, D'Amato FR. 1991. Anxiety and maternal aggression in house mice (Mus musculus): a look at interindividual variability. J Comp Psychol 105(3):295-301.

Magarinos AM, McEwen BS. 1995. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. Neuroscience 69(1):89-98.

Meaney MJ, Viau V, Aitken DH, Bhatnagar S. 1989. Glucocoticoid receptors in brain and pituitary of the lactating dam. Physiology& Behavior 45(1):209-12

Neumann ID. 2001. Alterations in behavioral and neuroendocrine stress coping strategies in pregnant, parturient and lactating rats. Prog Brain Res 133:143-52.

Neumann ID. 2003. Brain mechanisms underlying emotional alterations in the peripartum period in rats. Depress Anxiety 17(3):111-21.

Neumann ID, Johnstone HA, Hatzinger M, Liebsch G, Shipston M, Russell JA, Landgraf R, Douglas AJ. 1998. Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. J Physiol 508 ( Pt 1):289-300.

Oatridge A, Holdcroft A, Saeed N, Hajnal JV, Puri BK, Fusi L, Bydder GM. 2002. Change in brain size during and after pregnancy: study in healthy women and women with preeclampsia. AJNR Am J Neuroradiol 23(1):19-26.

Pawluski JL, Galea LA. 2006. Hippocampal morphology is differentially affected by reproductive experience in the mother. J Neurobiol 66(1):71-81

Pawluski JL, Galea LA. 2007. Reproductive experience alters hippocampal neurogenesis during the postpartum period in the dam. Neuroscience 149(1):53-67.

Pawluski JL, Charlier TD, Lieblich SE, Hammond GL,Galea LA. 2009. Reproductive experience alters corticosterone and CBG levelsin the rat dam. Physiology&Behavior 96(1):108-14

Pawluski JL, van den Hove DL, Rayen I, Prickaerts J, Steinbusch HW. 2011. Stress and the pregnant female: Impact on hippocampal cell proliferation, but not affective-like behaviors. Horm Behav 59(4):572-80

Pham K, Nacher J, Hof PR, McEwen BS. 2003. Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur J Neurosci 17(4):879-86.

Purcell RH, Sun B, Pass LL, Power ML, Moran TH, Tamashiro KL. 2011. Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior. Physiol Behav 104(3):474-9.

Robertson E, Grace S, Wallington T, Stewart DE. 2004. Antenatal risk factors for postpartum depression: a synthesis of recent literature. Gen Hosp Psychiatry 26(4):289-95.

Rosenbrock H, Koros E, Bloching A, Podhorna J, Borsini F. 2005. Effect of chronic intermittent restraint stress on hippocampal expression of marker proteins for synaptic plasticity and progenitor cell proliferation in rats. Brain Res 1040(1-2):55-63.

Russell JA, Douglas AJ, Ingram CD. 2001. Brain preparations for maternity--adaptive changes in behavioral and neuroendocrine systems during pregnancy and lactation. An overview. Prog Brain Res 133:1-38.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O and others. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301(5634):805-9.

Slattery DA, Neumann ID. 2008. No stress please! Mechanisms of stress hyporesponsiveness of the maternal brain. J Physiol 586(2):377-85.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA. 2011. Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476(7361):458-61.

Stern JM, Goldman L, Levine S. 1973. Pituitary-adrenal responsiveness during lactation in rats. Neuroendocrinology 12(3):179-91.

Takahashi T, Nowakowski RS, Caviness VS, Jr. 1992. BUdR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. J Neurocytol 21(3):185-97.

Tanapat P, Hastings NB, Reeves AJ, Gould E. 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrusof the adult female rat. J Neurosci 19(14): 5792-801

Tanapat P, Hastings NB, Rydel TA, Galea LA, Gould E. 2001. Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. J Comp Neurol 437(4):496-504.

Torner L, Karg S, Blume A, Kandasamy M, Kuhn HG, Winkler J, Aigner L, Neumann ID. 2009. Prolactin prevents chronic stress-induced decrease of adult hippocampal neurogenesis and promotes neuronal fate. J Neurosci 29(6):1826-33.

Varodayan FP, Zhu XJ, Cui XN, Porter BE. 2009. Seizures increase cell proliferation in the dentate gyrus by shortening progenitor cell-cycle length. Epilepsia 50(12):2638-47.

Walker CD, Trottier G, Rochford J, Lavallee D. 1995. Dissociation between behavioral and hormonal responses to the forced swim stress in lactating rats. J Neuroendocrinol 7(8):615-22.

Westenbroek C, Den Boer JA, Veenhuis M, Ter Horst GJ. 2004. Chronic stress and social housing differentially affect neurogenesis in male and female rats. Brain Res Bull 64(4):303-8.

FIGURES

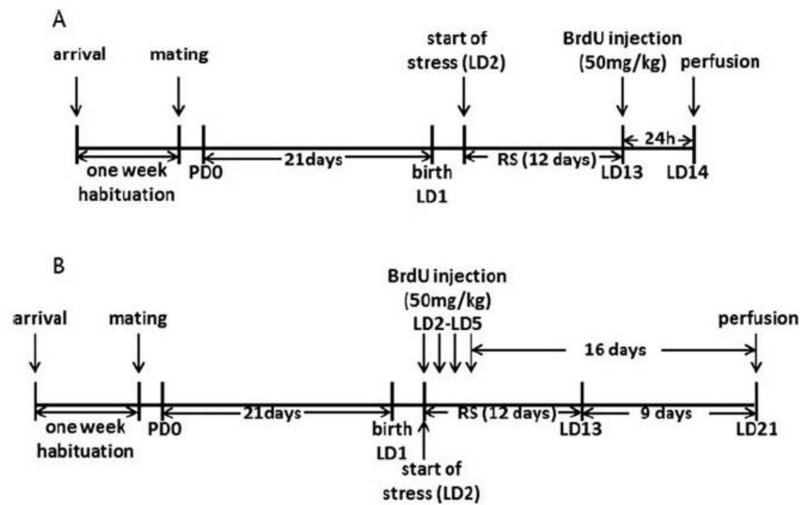


**Figure 1: Schematic representation of the temporal designs used in the present studies**

Temporal designs used to assess A) cell proliferation, immature neuron production (DCX positive cells), brain weight and hippocampal volume and B) cell survival and neuronal/ astroglial differentiation. After 1 week of habituation, all females were mated. After birth, animals were exposed to 2h of RS for 12 consecutive days from (LD2 to LD13, or equivalent in nulliparous animals). They received either A) a single BrdU injection (50mg/kg; i.p.) on the last day of stress (LD13 or equivalent in nulliparous animals) or B) four BrdU injections (50mg/kg; i.p.) on four consecutive days ((LD2-LD5 or equivalent in nulliparous animals/day 1- day 4 of stress exposure). Animals were intra-cardiacally perfused either A) 24h after (LD14 or equivalent in nulliparous animals) or B) 16 days after (LD21 or equivalent in nulliparous animals) the last BrdU injection.

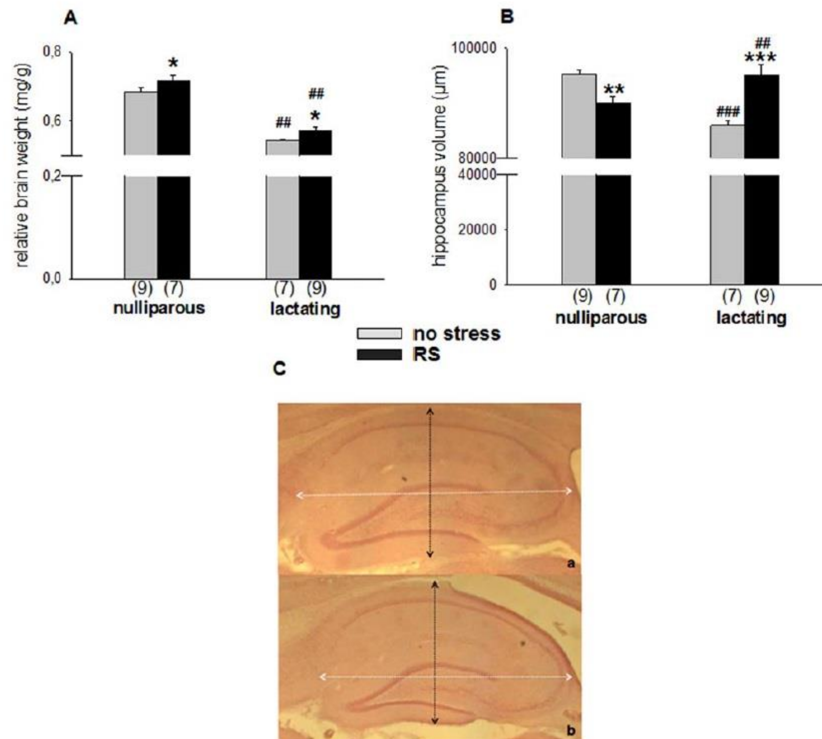


**Figure 2: Effect of reproductive status and stress on relative brain weight and hippocampal volume**

The relative brain weight (A) (experimental timeline see *Figure 1A*) and the hippocampal volume (B) (experimental timeline see *Figure 1A*) was assessed in the DG of the hippocampus in nulliparous and lactating females under basal (grey bars) or repeated stress (black bars) conditions. A) Lactating animals showed a lower relative brain weight under basal conditions; stress during lactation led to an increase in relative brain weight, when compared with the respective non-stressed group B) Analysis of hippocampal volume revealed a reduced hippocampal volume in lactating animals under basal conditions; stress led to a reduced volume in nulliparous animals, whereas it had the opposite effect in lactating animals. C) Representative image of the Cresyl violet staining for the analysis of hippocampal volume ( a) nulliparous non-stressed animal; black dotted arrow= 242µm, white dotted arrow=466µm b) lactating non-stressed animals; black dotted arrow= 230µm, white dotted arrow=420µm). Data represent mean ± SEM with the numbers in parentheses representing the group sizes. *, p<0.05 *vs.* respective non-stressed group; **, p<0.01 *vs.* respective non-stressed group; ***, p<0.001 *vs.* respective non-stressed group; ##, p<0.01 *vs.* respective nulliparous group; ###, p<0.001 *vs.* respective nulliparous group; RS, restraint stress

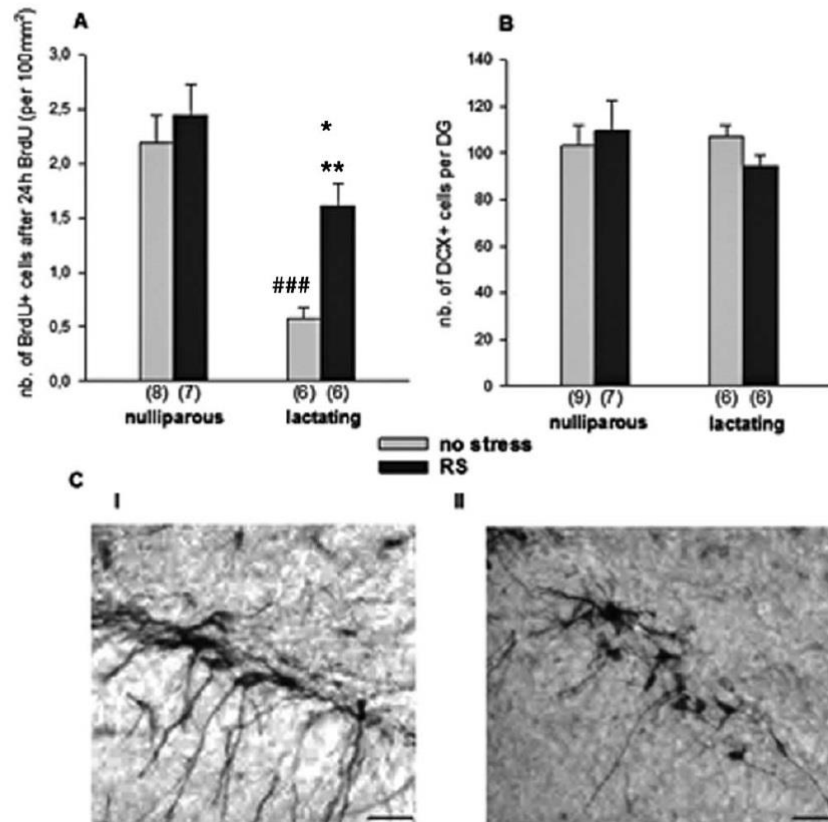


**Figure 3: Effect of reproductive status and stress on cell proliferation and immature neuron production in the DG**

The total number of proliferating cells (A) (experimental timeline see *Figure 1A*) and the number of immature neurons (DCX+ cells) (B) (experimental timeline see *Figure 1A*) was assessed in the DG of the hippocampus in nulliparous and lactating females under basal (grey bars) and stress (black bars) conditions. A) Lactating animals showed a lower number of proliferating cells, both under basal and repeated stress conditions; stress during lactation led to an increase in cell proliferation when compared with the respective non-stressed group B) There was no effect of reproductive status or stress on immature neuron production; C) Representative image of a DAB-immunostaining against DCX (lactating non-stressed animal (I) and lactating stressed animal (II)), scale bar = 50µm. 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 nulliparous group; ###, $p<0.001$ *vs.* respective nulliparous group; RS, restraint stress

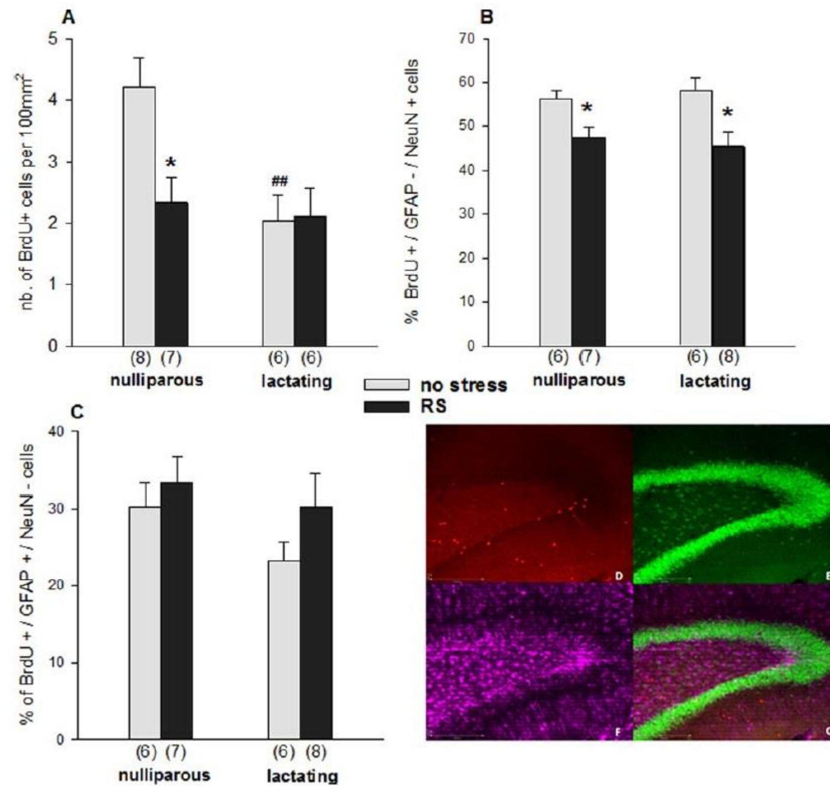


**Figure 4: Effect of reproductive status and stress on cell survival and differentiation patterns in the DG**

The number of surviving cells (A) (experimental timeline see *Figure 1B*) and the percentage of cells differentiating into neurons (BrdU positive, GFAP negative and NeuN positive cells) (B) (experimental timeline see *Figure 1B*) and astroglial cells (BrdU positive, GFAP positive, NeuN negative cells) (C) (experimental timeline see *Figure 1B*) was assessed in the DG of the hippocampus in nulliparous and lactating females under basal (grey bars) or stress (black bars) conditions. A) Lactating animals showed a reduction in cell survival under non-stress conditions; stress led to a reduction in cell survival in nulliparous, but not lactating animals. B) Repeated stress reduced neuronal differentiation in nulliparous and lactating females, no basal differences were observed between nulliparous and lactating animals. C) There was no effect of the reproductive status or stress on astroglial differentiation patterns. D-G) Representative images of the triple-immunfluorescent image (lactating non-stressed animal) with antibodies against BrdU (4D) (red), GFAP (4E) (violet) NeuN (4F) (green) and merge (4G), scale bar = 50µm. 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 nulliparous group. RS, restraint stress

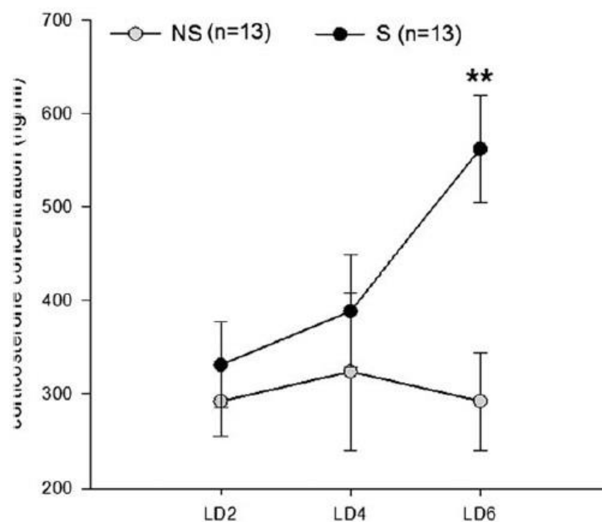


**Figure 5: Effect of reproductive status and stress on plasma CORT levels**

Plasma CORT levels on LD2, LD4 and LD6 were assessed immediately prior to 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

TABLE

| | Status effect | Stress effect | Status × Stress effect |
|---|---|---|---|
| Absolute brain weight | $F_{(1,28)} = 10.81$, $P < 0.001$* | | |
| Relative brain weight (Fig. 2A) | $F_{(1,28)} = 84.83$, $P < 0.0001$* | $F_{(1,28)} = 5.31$, $P < 0.01$* | |
| Hippocampal volume (Fig. 2B) | | | $F_{(1,27)} = 29.72$, $P < 0.0001$* |
| Cell proliferation (Fig. 2D) | $F_{(1,23)} = 26.45$, $P < 0.0001$* | $F_{(1,23)} = 7.40$, $P < 0.05$* | |
| Immature neurons (Fig. 3A) | $F_{(1,24)} = 0.46$, $P = 0.505$ | $F_{(1,24)} = 0.13$, $P = 0.723$ | |
| Cell survival (Fig. 3B) | | | $F_{(1,23)} = 4.47$, $P < 0.05$* |
| Neuronal differentiation (Fig. 4A) | $F_{(1,23)} = 0.001$, $P = 0.972$ | $F_{(1,23)} = 11.54$, $P < 0.01$* | |
| Astroglial differentiation (Fig. 4B) | $F_{(1,23)} = 2.32$, $P = 0.142$ | $F_{(1,23)} = 2.32$, $P = 0.142$ | |
| Corticosterone levels (Fig. 5) | | $F_{(1,28)} = 9.04$, $P < 0.01$* | |

**Table 1. Overall statistical effects for Figures 2, 3, 4, and 5**

One- or two-way analysis of variance (ANOVA) with or without repeated measures was applied a appropriate followed by Fisher's *post hoc* test. * $P < 0.05$