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Sex-dependent regulation of hippocampal neurogenesis under basal and chronic stress conditions in rats

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Running title: Regulation of hippocampal neurogenesis in males and females

Keywords: stem cell quiescence, neurons, differentiation, spatial memory, corticosterone

Disclosure:

This manuscript is the peer-reviewed version of the article 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 23(6):476-87.
doi: 10.1002/hipo.22107. Epub 2013 Mar 18.
The final publication is available at: http://onlinelibrary.wiley.com/doi/10.1002/hipo.22107/epdf

ABSTRACT

Sex differences in basal as well as in stress-induced hippocampal neurogenesis processes have been reported in the literature. However, studies directly comparing sex differences on multiple neurogenesis processes under such conditions are lacking to date. Therefore, the aim of the present study was to directly compare cell proliferation and survival, neuronal and astroglial differentiation as well as stem cells quiescence in male and female Wistar rats under both basal and chronic stress conditions (12 days of 2h restraint stress (RS)). In addition, corticosterone (CORT) levels and spatial working memory were assessed. Under baseline conditions, only the number of immature neurons within the hippocampal dentate gyrus was higher in males compared with females. In contrast, chronic stress resulted in a number of sex-specific alterations. Thus, stress exposure reduced cell proliferation in males with a concurrent increase in stem cell quiescence, while it did not alter either parameter in females but decreased cell survival in females. 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. While basal CORT levels were higher in females, chronic stress exposure did not affect this parameter in either sex across the initial stress period. This study presents the first direct and detailed evaluation of sex-dependent and chronic stress-induced changes in adult hippocampal neurogenesis not only showing changes in cell proliferation and survival but moreover immature neuron production, differentiation patterns, stem cell quiescence and therefore contributes to a better understanding of sex differences in neurogenesis processes.

INTRODUCTION

It is now a well-established fact that new nerve cells are produced throughout the lifespan in the brain of different species including 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 subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles. 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 has been shown to be relevant for learning and memory and *vice versa* (for reviews see Koehl and Abrous, 2011; Leuner et al., 2006). 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; Lucassen et al., 2010; Sahay and Hen, 2007).

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. In addition, females have been shown to express a greater number of mossy fibre synapses in the CA3 region compared to males (Madeira et al., 1991). Moreover, 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 sex steroids during the development and adulthood (McEwen et al., 1995). Ovarian hormones, 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).

Another important factor that affects adult neurogenesis is stress and it is well-known that males and females exhibit different stress sensitivity and coping mechanisms (Bowman et al., 2001; Dalla et al.,

2008; Wolf et al., 2001). In comparison to males, who habituate to stressful situations, females show a longer and more robust stress-induced rise in CORT levels (Falconer and Galea, 2003; Galea et al., 1997; Neumann et al., 2000). 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 differences in stress-coping and stress-response, 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; Shors et al., 2007; Silva et al., 2008; Westenbroek et al., 2004) without affecting cell survival (Joels et al., 2004), induces apical dendritic atrophy of CA3 pyramidal neurons (Galea et al., 1997; Magarinos and McEwen, 1995; Uno et al., 1989) 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 to the same extent as in males (Galea et al., 1997; Shors et al., 2007; Uno et al., 1989; Westenbroek et al., 2004). 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/ maintenance of stem cells is missing so far. Moreover, those studies that have been performed to date have focussed on either male or female rodents, which further complicates the assessment of sex-related differences in neurogenesis.

Therefore, the main aim of our study was to expand the knowledge about basal and stress-induced sex differences in cell proliferation and cell survival provided by previous work of Westenbroek et al. to more complex parameters like cell fate and quiescence. Furthermore, basal and stress-induced plasma CORT levels, as well as spatial working memory in the Y-maze were assessed.

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 7 days after arrival. Thereafter, 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.

Experimental cohorts

A total of three different experimental cohorts were used for the present studies. One cohort of animals (female n= 15; male n= 13) was used to analyze hippocampal cell proliferation and immature neurons (see also *Figure 1A*), one cohort (female n= 13; male n= 12) was used to analyze hippocampal cell survival, cell fate, quiescence and spatial memory (see also *Figure 1B*) and a further cohort of animals (female n= 18; male n= 18) was used to assess plasma CORT levels.

Chronic stress procedure

Animals of stressed groups were subjected to 2h restraint stress (RS), which is an effective stressor in male and female rats (Barha et al., 2011; Magarinos and McEwen, 1995; Pham et al., 2003) for 12 consecutive days between 10:00 - 12:00 A.M.; a protocol that has previous been shown to effect neurogenesis (Barha et al., 2011). 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. The body weight of each animal was recorded daily 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; Sigma, Steinheim, Germany; (50mg/kg, i.p., 10mg/ml in 0.9% NaCl)) once, on the last day of stress, immediately after removal from the restraint tubes, i.e. at 12:00 A.M (Kandasamy et al., 2010). 24h 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 (Kandasamy et al., 2010). 24h 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 4 days of stress exposure (days 1-4; immediately after RS) as described above. 16 days after the last BrdU injection, and 9 days after the last RS session (day 20) rats were anesthetized and perfused as described above.

BrdU was freshly prepared in 0.9% NaCl solution to a dilution of 10mg/ml on each injection day. (For schematic representation of the temporal design of the study 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 a 30% sucrose solution (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 (Leica Microsystems, Wetzlar, Germany) 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 as previously described (Kandasamy et al., 2010). 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

30min. For DNA denaturation, sections were incubated for 2h in 50% formaldehyde/2 X saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, rinsed for 5min in 2 X SSC, incubated in 2M HCl for 30min 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 1h, 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 1h, rinsed for 5min in 2 X SSC, incubated in 2M HCl for 30min at 37°C and washed for 10min in 0.1M boric acid, pH 8.5. Thereafter, sections were washed four times in TBS for 5min, before they were incubated with FSGB for 30min, 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 $100mm^2$ 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). Z-stacks were built using the Volocity Software (Perkin Elmer). 50 BrdU-positive labelled cells per animal were analyzed 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) as previously described (Kandasamy et al., 2010).

Experiment 3: Effect of stress 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 under basal and stress conditions. Therefore, jugular vein surgery was performed as previously described 5 days prior to the

start of the experiment (Hillerer et al., 2011; 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 (30,000IU/ml; Centravet, Bad Bentheim, Germany). On the first, third and five days of chronic stress procedure the catheter was attached to an extension tubing connected to a 1-ml plastic syringe filled with sterilized heparinised 0.9% saline (30 IU/ml, Heparin-Natrium, Ratiopharm, Ulm, Germany) at 07:30 and each rat was then left undisturbed for 2h. Two basal samples (basal sample one: 0.6ml and basal sample two: 0.2ml) were taken 30min apart and were used to calculate the mean basal concentrations for CORT for each animal. The mean CORT concentration of male non-stressed animals on day 1 (397.6 ng/ml) was set as 100% and all other values calculated to this mean. After the sampling of the second basal sample rats were placed into the restraint tubes. A stress sample (0.6ml) was taken 30min after the start of the stress procedure. After 2h 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 analyzed for CORT using a commercially available ELISA kit (DRG Instruments GmbH, Marburg, Germany).

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 connected 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 procedure, in animals that were also used for the analysis of cell survival, differentiation and quiescence (see also *Figure 1B*) (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 10min 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 analyzed using 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 separate for number of cells in the dentate gyrus with group (male/ female and control/ stress) as between subject factors. Any statistical differences, which were set at $p < 0.05$, were further analyzed using a Fisher's *post-hoc* test. Statistical analyses were performed using SPSS for Windows (version 18; SPSS Inc, Chicago, IL, USA). Overall statistics are shown in *Table 1*. Due to technical issues some sections could not be analyzed (proliferation: one female non-stressed animal and one male stressed animal; DCX: one female non-stressed animal; quiescence: one female non-stressed animal).

RESULTS

Basal and chronic stress-induced sex differences in cell proliferation (Experiment 1)

To assess sex differences in cell proliferation under basal as well as chronic stress conditions animals were given a single BrdU injection after 12 days RS and perfused 24h later (see *Figure 1A*). The 12-day RS exposure decreased the number of BrdU positive cells specifically in males (Fig. 2A; $p<0.01$), while there was no effect on cell proliferation in females. Moreover, the number of proliferating cells in stressed males was below the one of the respective female group (Fig. 2A; $p<0.05$). No differences in basal cell proliferation between males and females were observed ($p>0.05$; Fig 2A).

Basal and chronic stress-induced sex differences in stem cell quiescence (Experiment 2)

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 RS exposure (or equivalent in non-stressed groups) and were perfused 16 days later (see *Figure 1B*). Quantitative confocal analysis revealed an increase in the number of quiescent stem cells in the DG of stressed males compared to the respective non-stressed group (Fig. 2B; Mann Whitney *U*; $p=0.01$;). Consistent with the increase in quiescence, the number of BrdU / SOX 2 / PCNA triple-positive cells was reduced in stressed compared to unstressed males (Fig. 2C; Mann Whitney *U*; $p=0.004$). However, there were no differences in these parameters between males and females in either basal or stressed conditions. Figure 2D shows a representative image of a BrdU / SOX2 / PCNA triple immunolabeling.

Basal and chronic stress-induced sex differences on immature neuron production (Experiment 1)

Next we questioned if the stress-associated effects on hippocampal cell proliferation in males translated into different numbers of immature neurons. Therefore, animals were given a single BrdU injection after 12 days RS and perfused 24h later (see *Figure 1A*). The number of DCX positive cells in the DG was analyzed. Surprisingly, the number DCX positive cells in males was higher than in females irrespective of the stress exposure (Fig.3A; $p<0.05$). Figure 3B shows a representative image of DCX immunoreactive cells in the DG.

Basal and chronic stress-induced sex differences on cell survival (Experiment 2)

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 1B*). Males showed a lower cell survival compared to females under basal conditions ($p<0.001$; Fig. 3C), as well as a reduction in the number of surviving cells in females after chronic stress exposure ($p<0.01$; Fig. 3C). However, no effect of stress on cell survival was observed in males ($p>0.05$; Fig. 3C).

Basal and chronic stress-induced sex differences on neuronal and astroglial differentiation (Experiment 2)

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), were perfused 16 days later (see *Figure 1B*), and the hippocampus was analyzed for cells double-positive for BrdU and NeuN for neuronal differentiation or BrdU and GFAP for astroglial differentiation. Stressed females displayed a reduction in the percentage of neuronal differentiation compared with the respective non-stressed group (Fig. 4A; Mann Whitney *U*; $p=0.022$). Astroglial differentiation was shown to be independent of stress and of sex (Fig. 4B). Figure 4C shows a representative image of BrdU / NeuN / GFAP triple labelling of the DG.

Basal and chronic stress-induced sex differences on plasma CORT levels (Experiment 3)

To examine the effect of chronic stress on basal and stress induced CORT levels, blood samples were taken immediately prior and during stress exposure on day 1, 3 and 5 of the 12-day stress paradigm. The mean CORT concentration of male non-stressed animals on day 1 (397.6 ng/ml ± 104.4ng/ml) was set as 100% and all other values were calculated based on this mean. There were no differences in basal CORT levels between non-stressed and stressed groups in either sex across all tested days. Detailed analyses of day five CORT levels (the day after the last BrdU injection for the survival study) showed that females had higher basal CORT levels compared with males independent of the stress

condition (Fig. 5). Moreover, stressed groups showed a significant rise in CORT after 30min of stress compared to baseline CORT levels (Fig. 5), which was also the case on days 1 and 3 (data not shown).

Basal and chronic stress-induced sex differences on spatial working memory (Experiment 4)

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. No significant effects of sex or stress on the percentage of alternations in the Y-maze were observed (data not shown).

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 hippocampal cell proliferation, which was associated with elevated stem cell quiescence, while neither parameter was altered by stress in females. In contrast, 16-days cell survival under basal conditions was higher in females than in males, and chronic stress exposure impaired this process (decreased) specifically in females. Immature neuron production was higher in males than in females under basal and chronic stress conditions, whereas chronic stress exposure did not affect this parameter in either sex. 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, neither basal CORT levels nor spatial working memory were affected by stress exposure in either sex. 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 and cell survival, but moreover sheds light on detailed neurogenesis processes like immature neuron production, differentiation patterns and stem cell quiescence.

Chronic stress impairs hippocampal neurogenesis in numerous species, with the effects dependent upon the intensity and frequency of stress exposure as well as the timing of BrdU injection (Czeh et al., 2002; Gould et al., 1997; Gould et al., 1998; Pham et al., 2003; Tanapat et al., 2001; Torner et al., 2009; Uno et al., 1989) (for review see Abrous et al., 2005). Validating the efficiency of our stress model, which has previously been used in neurogenesis studies (Barha et al., 2011; Magarinos and McEwen, 1995; Pham et al., 2003), we found a decreased body weight gain in males and females (see also *Table 2*), 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 (Conrad et al., 1996; Galea et al., 1997; Magarinos and McEwen, 1995).

In accordance with previous work (Heine et al., 2004; Madeira and Paula-Barbosa, 1993; Madeira et al., 1991; Pham et al., 2003), we revealed that chronic stress exposure attenuated cell proliferation in males. Although previous stress studies have shown an increase in cell proliferation in females (Pawluski et al., 2011), this parameter was unaffected in our studies; despite basal levels not differing (Westenbroek et al., 2004). This discrepancy might be either due to the use of another cell cycle marker (Ki-67) or diverging stress protocols. However, to date, no studies have assessed basal or stress-induced differences in stem cell quiescence. We could show that under non-stressed conditions males and females expressed similar numbers 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 and lack of effect in females. 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 mechanisms 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. Transforming growth factor beta might be one such candidate, which has been shown to induce cell cycle arrest in neural progenitor cell cultures (Kandasamy et al., 2010).

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 (Barha et al., 2011; Falconer and Galea, 2003; Galea et al., 1997; Westenbroek et al., 2004). 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 (Heine et al., 2004; Madeira and Paula-Barbosa, 1993; Madeira et al., 1991; Pham et al., 2003; Westenbroek et al., 2004), and mirror the observed changes in neuronal differentiation patterns. Although, previous studies revealed neuronal differentiation to average around 70% of BrdU positive cells, the number of NeuN expressing cells in our study was rather about 50-60%. One reason for this apparent discrepancy might be the time point of assessing differentiation patterns. Whereas most of survival studies use a four- week time point after the last BrdU labelling, we assessed neuronal and astroglial differentiation already 16-21 days after the last BrdU injection, given the experimental outline of the study. Therefore, it might be that some of the cells were not fully differentiated at the time of assessment.

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, which reflects 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 (Becker and Grilo, 2007; Kendler, 1998; Shors and Leuner, 2003) and the fact that there seems to be a link between a reduced neurogenesis and depression (Czeh et al., 2001; Oomen et al., 2007; Santarelli et al., 2003; 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; Hellsten et al., 2002; Lemaire et al., 1999; Tanapat et al., 2001). In the present study, we demonstrate that females exhibit not only higher basal CORT levels than males, but also showed higher peak CORT responses during RS than males (Romeo et al., 2004).

It is astonishing that, while chronic stress did not affect cell proliferation in the DG of female rats, 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 basal 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 (Neumann et al., 2000; Pi and Grattan, 1999; Torner et al., 2004) and counteract stress-induced changes in neurogenesis (Leuner et al., 2010; Torner et al., 2009). Therefore, future studies will assess the involvement of these neuropeptides on the observed sex differences in various aspects of 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; Witter, 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., 1993). Thus, it is possible that CORT may rather act indirectly 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 (Reagan and McEwen, 1997; Stein-Behrens et al., 1994) or release of growth factors (Kuhn et al., 1997).

Learning and memory processes, especially spatial memory, have consistently been linked to adult hippocampal neurogenesis (Kee et al., 2007; Leuner et al., 2006; Riedel et al., 1999; Saxe et al., 2006; Shors et al., 2001; 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 of functional relevance 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 (Dalla et al., 2009; Dalla and Shors, 2009; de Frias et al., 2006; Shors, 2006), 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 in the Y-maze. Maybe other tests for spatial working memory like the Morris water maze, which is frequently used to assess correlations between neurogenesis and spatial working memory, would have provided a different result and will therefore be added in subsequent studies. Moreover, as stress and sex had multiple effects at different levels of neurogenesis, it might well be that some of the deficits encountered might be compensated by modulations at a different stage of neurogenesis and thus not translate into a behavioral difference.

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 (Dagyte et al., 2011; Revest et al., 2009). Therefore, future studies will assess anxiety-related behavior to reveal a potential link between differences in the number of immature neurons and this type of affective behavior in males and females.

CONCLUSIONS

The results of the present study indicate that as well as basal differences, exposure to chronic RS alters the neurogenic 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.

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ACKNOWLEDGMENTS

We thank Dr. Mahesh Kandasamy for introduction into the several staining techniques and Rodrigue Maloumby, Andrea Havasi and Gabriele Schindler 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), 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), and the state of Salzburg (to L.A.).

FIGURES

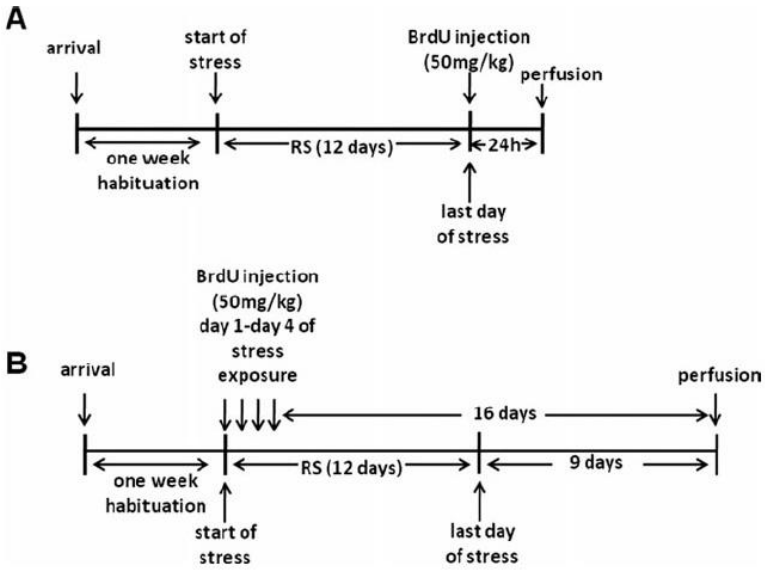

Figure 1: Schematic representation of the temporal designs used in the present studies

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

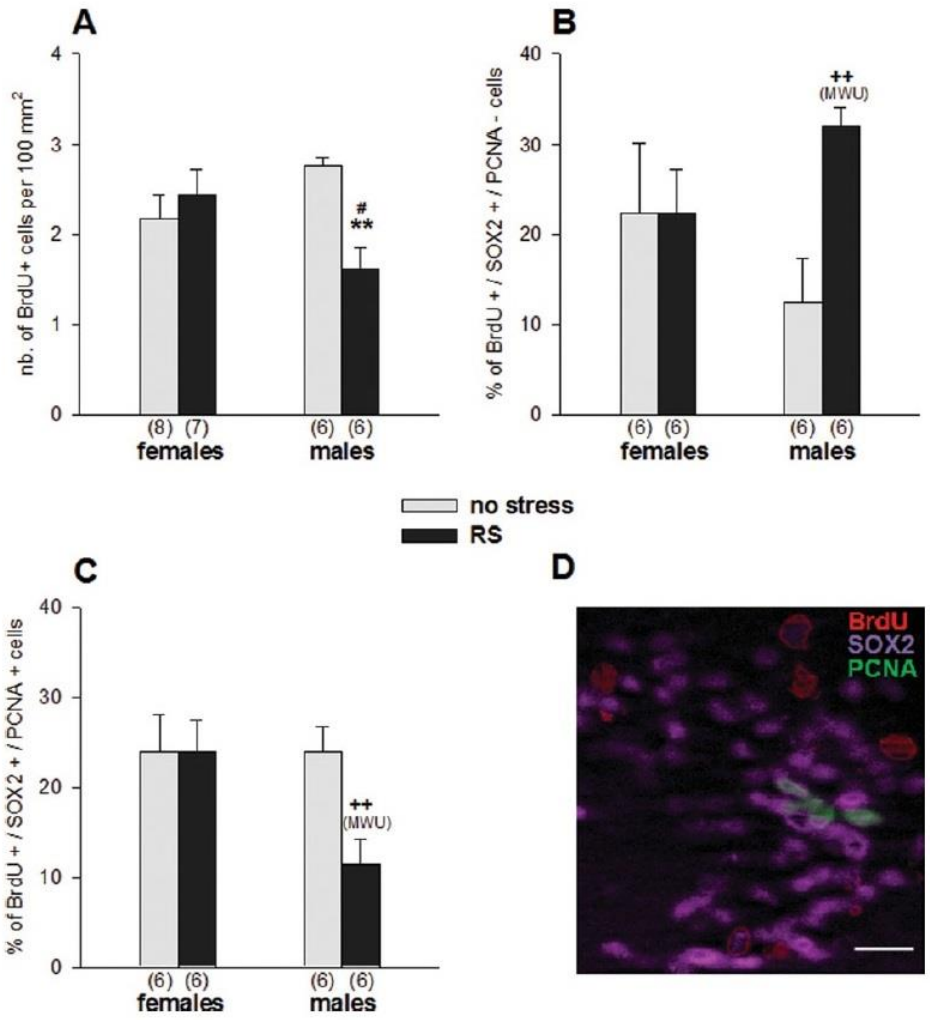

Figure 2: Effect of sex and chronic stress on cell proliferation and stem cell quiescence in the DG

The total number of proliferating cells (A) (experimental timeline see *Figure 1A*), the percentage of quiescent stem cells (B) (experimental timeline see *Figure 1B*) and percentage of proliferating stem cells (C) (experimental timeline see *Figure B*) 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), 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 female group; ++, $p<0.01$ *vs.* respective non-stressed group using a Mann Whitney *U* test (MWU). NS, non-stressed; S, stressed

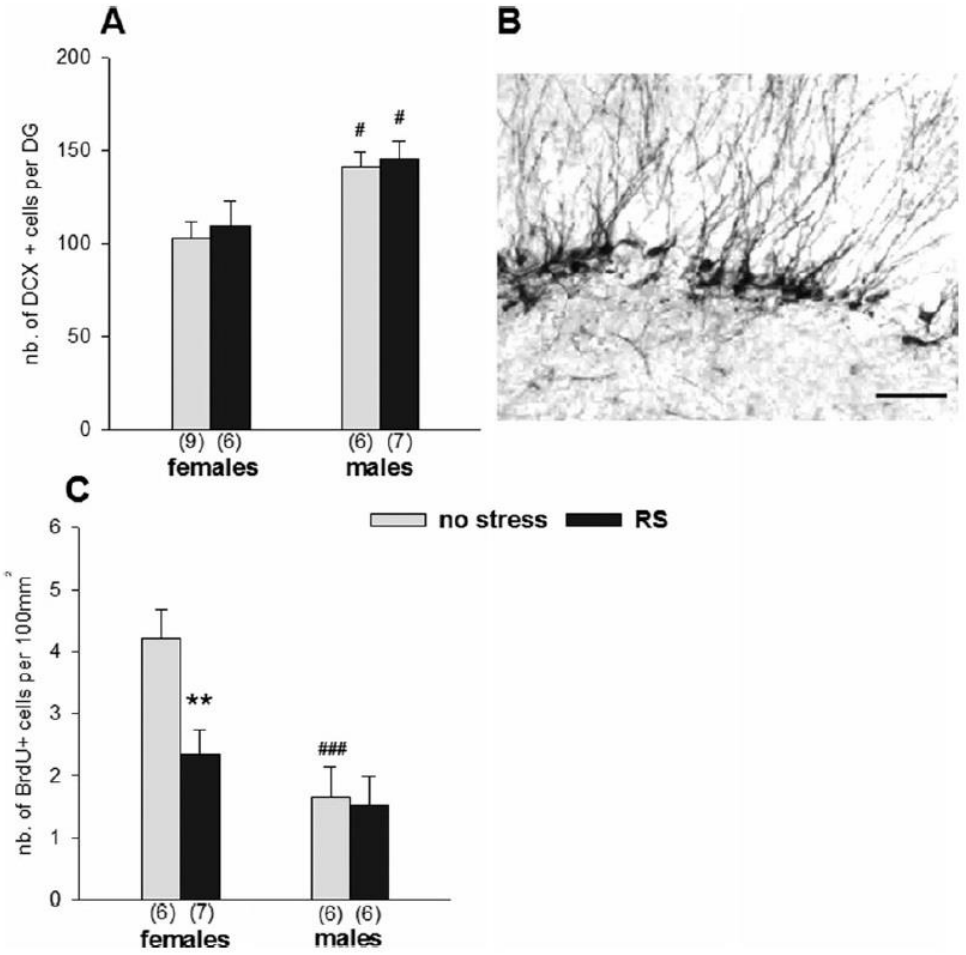

Figure 3: Effect of sex and chronic stress on immature neuron production and survival of cells

The number of immature neurons (DCX+ cells) (A) (experimental timeline see *Figure 1A*) and the number of surviving cells (C) (experimental timeline see *Figure 1B*) 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) Representative image (male non-stressed animal) of a DAB-immunostaining against DCX, scale bar = 50µm. C) 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. 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

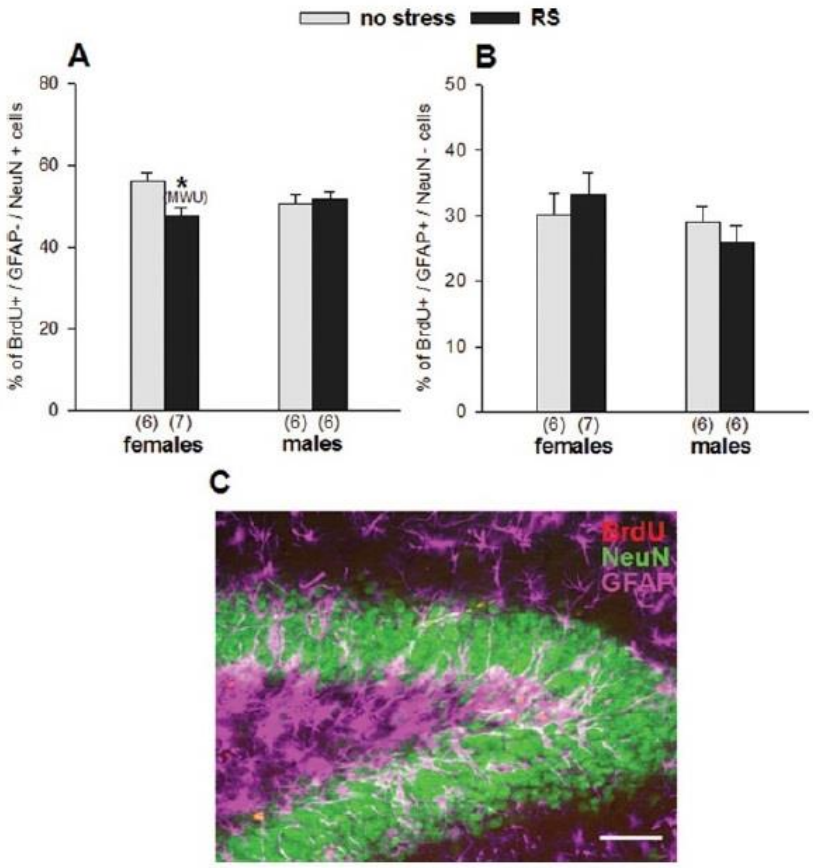

Figure 4: Effect of sex and chronic stress on differentiation patterns

The percentage of cells differentiating into neurons (BrdU positive, GFAP negative and NeuN positive cells) (A) (experimental timeline see *Figure 1B*) and astroglial cells (BrdU positive, GFAP positive, NeuN negative cells) (B) (experimental timeline see *Figure 1B*) 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), scale bar = 50µm. 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

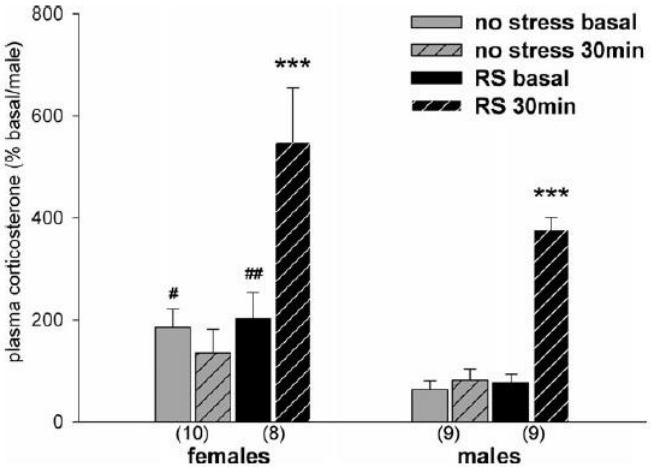

Figure 5: Effect of sex and chronic stress exposure on basal CORT levels

Basal (filled bars) and 30min stress (striped bars) CORT levels on day 5 were assessed. Females showed higher basal CORT levels on day 5 compared to the respective male group. Stress induced an increase in CORT levels when compared with basal CORT values. Data represent mean ± SEM (% CORT concentration compared with M/NS group on day 1) with the numbers in parentheses representing the group sizes. #, $p<0.05$ *vs.* respective male group, ##, $p<0.01$ *vs.* respective male group, *** $p<0.001$ *vs.* basal level. NS, non-stressed; S, stressed

TABLES

	Sex effect	Stress effect	Sex × Stress effect
Cell poliferation (Fig 2A)			$F_{(1,23)} = 8.504$; $P = 0.008^*$
Quiescent stem cells (Fig 2B)	$F_{(1,20)} = 2.032$; $P = 0.914$	$F_{(1,20)} = 3.873$; $P = 0.063$	
Proliferating stem cells (Fig 2C)	$F_{(1,20)} = 3.876$; $P = 0.062$	$F_{(1,20)} = 3.980$; $P = 0.063$	
Immature neurons (Fig 3A)	$F_{(1,24)} = 12.52$; $P = 0.02^*$	$F_{(1,24)} = 0.275$; $P = 0.605$	
Cell survival (Fig 3C)	$F_{(1,21)} = 4.867$; $P = 0.039^*$	$F_{(1,21)} = 13.485$; $P = 0.001^*$	
Neuronal differentiation (Fig 4A)	$F_{(1,21)} = 0.09$; $P = 0.768$	$F_{(1,21)} = 2.118$; $P = 0.160$	
Astroglial differentiation (Fig 4B)	$F_{(1,21)} = 1.795$; $P = 0.195$	$F_{(1,21)} = 0.000$; $P = 0.99$	
Corticosterone levels (Fig 5)	$F_{(1,30)} = 17.916$; $P = 0.001^*$	$F_{(1,30)} = 33.856$; $P = 0.001^*$	

Table 1: Overall effects for Figures 2, 3, 4 and 5

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

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
F/NS (9)	0	1.5	−0.7	1.9	4.0	2.3	1.9	2.3	5.8	4.3	3.3	6.5
	0	*1.16*	*1.85*	*1.28*	*1.59*	*1.53*	*1.24*	*1.41*	*1.71*	*1.8*	*1.92*	*1.91*
F/S (7)	0	−4.4	−7.4*	−9.7*	−10.0*	−9.4*	−12.6*	−11.9*	−12.9*	−12.1*	−12.6*	−11.3*
	0	*2.6*	*3.0*	*3.0*	*2.77*	*3.4*	*3.54*	*3.46*	*3.62*	*3.89*	*4.65*	*4.44*
M/NS (6)	0	1.7	6.2	9.5	9.5	12.8	15.5	15.8	19.2	22.3	26.0	30.0
	0	*3.27*	*1.62*	*1.52*	*1.86*	*1.7*	*1.93*	*1.66*	*2.33*	*1.2*	*1.69*	*1.41*
M/S (7)	0	−5.2	−9.0*	−13.0*	−14.5*	−17.5*	−17.5*	−19.7*	−18.8*	−19.7*	−18.3*	−20.0*
	0	*3.13*	*2.34*	*2.46*	*2.49*	*2.95*	*2.03*	*3.44*	*2.89*	*2.91*	*3.05*	*1.91*

Table 2: Average body weight gain in (g) and SEM in (g)

Average body weight gain in (g) was assessed during the 12 days of chronic stress exposure (experimental timeline see *Figure 1 A*). Data represent mean ± SEM (in italics) with the numbers in parentheses representing the group sizes, * significant p-value *vs.* respective non-stressed group. d, day; F, female; M, Male; NS, non-stressed; S, stressed