Adult hippocampal cell birth and death in relation to stress, aging and the vasculature

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Chapter 2

Suppressed Proliferation and Apoptotic Changes in the Rat Dentate Gyrus after Acute and Chronic Stress are Reversible

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Abstract

Acute stress suppresses new cell birth in the hippocampus in several species. Relatively little is known, however, on how chronic stress affects the turnover, i.e. proliferation and apoptosis, of the rat dentate gyrus (DG) cells, and whether the stress effects are lasting. We questioned how 3-weeks of chronic unpredictable stress would influence the structural dynamic plasticity of the rat DG, and studied newborn cell proliferation, survival, apoptosis, volume and cell number in 10 week-old animals. To study lasting effects, another group of animals was allowed to recover for 3 weeks. Based on 2 independent parameters, BrdU and Ki-67 immunocytochemistry, our results show that both chronic and acute stress decrease new cell proliferation rate. The reduced proliferation after acute stress normalized already within 24 hrs. Interestingly, chronically stressed animals showed recovery after 3 weeks, albeit with still less proliferating cells than controls. Apoptosis on the other hand, increased after acute, but decreased after chronic stress. These results demonstrate that, although chronic stress suppresses proliferation and apoptosis, 3 weeks of recovery again normalized most of these alterations. This may have important implications for our understanding of the reversibility of stress-related hippocampal volume changes, as e.g. occur in depression.
Introduction

Stressful experiences, which elevate circulating glucocorticoids (GCs) levels, can affect hippocampal structure and function (Lopez et al., 1999; Joels, 2001; McEwen, 2001). GC excess e.g. modulates hippocampal excitability (Joels, 1997), long-term potentiation (Kim & Diamond, 2002) and learning (Bodnoff et al., 1995). In addition, chronic stress can affect structural hippocampal parameters, such as cell number (Sapolsky et al., 1985), the extent of the dendritic tree of pyramidal neurons (Magarinos et al., 1996; Galea et al., 1997; Lupien & McEwen, 1997) as well as functional characteristics in the CA3 region (Kole et al., 2002; Pavlides et al., 2002).

Recently, application of chronic unpredictable stress was shown to have clear synaptic effects in the DG too. Karst and Joels (2003) showed that chronic stress enhances the synaptic excitation of DG cells when corticosteroid levels rise. Alvarez et al. (2003) showed that the same paradigm dramatically reduces synaptic long-term potentiation (LTP) in the DG. Earlier it was already shown that chronic stress also affects cytogenesis in the dentate gyrus (DG) (Gould & Tanapat, 1999). Thus chronic stress not only affects the pyramidal, but also the granule cell signal transfer.

The DG is a unique brain area where neurogenesis and apoptosis occur during adulthood. DG turnover is a.o. controlled by circulating GC levels. Removal of glucocorticoids by adrenalectomy (ADX) increases the numbers of proliferating and apoptotic cells (Sloviter et al., 1989; Woolley et al., 1990; Gould & McEwen, 1993), whereas adult proliferation is reduced when GC levels are increased after acute, often psychosocial, stressors (Gould et al., 1997; Fuchs et al., 2001; Tanapat et al., 2001); (Gould et al., 1998; Czeh et al., 2002). Notably, these effects occur also in other species like tree shrew and marmoset (Gould et al., 1997; Gould et al., 1998) and can be more profound and long lasting when applied early in life (Tanapat et al., 1998; Lemaire et al., 2000).

Although the effect of acute stress on proliferation in the rat DG is well known, relatively little is known about how chronic stress affects the turnover of the DG cells. In theory, proliferation rate could habituate following chronic stress or after stressor repetition (Magarinos & McEwen, 1995). In the present study, we therefore questioned which structural changes are induced in the rat DG after 3 weeks of chronic unpredictable stress. Using two independent measures, Bromodeoxyuridine (BrdU) and Ki-67 immunocytochemistry, we stereologically assessed proliferation and survival of newborn cells. We also measured apoptosis and structural DG parameters such as volume.
and cell number, in 10-week old rats. Furthermore, since it is presently unclear whether the reductions in proliferation after chronic stress are lasting, or rather reversible, we also studied another group of animals that was allowed to recover for 3 more weeks. Similarly, for acute stress, changes in cell birth and death were examined using BrdU injections given prior to, and one day after animals were subjected to a one day stress paradigm.

**Material & Methods**

**Animals**

All animals were male Wistar rats (Harlan, the Netherlands) studied at the age of 10 weeks. Two rats were housed together under controlled conditions (21°C room temperature, 60% humidity, lights on from 8:00-20:00h) with food and water available ad libitum. In both the chronic and acute stress protocols, rats were randomly assigned to the control (n = 16), stressed (chronic n = 10; acute n = 12) or stress + recovery (n = 10) group. All experiments were approved by the local animal ethical committee of the University of Amsterdam.

**BrdU labeling**

To visualize newborn cells, animals were injected in the morning with BrdU (i.p., 10 mg/ml dissolved in 0.007 N NaOH/0.9% NaCl) at a dose of 200 mg/kg (Cameron & McKay, 2001). In order to study stress effects on proliferation and migration/survival of the newborn cells, the animals were studied after a survival time (S) of 24 hours, or 3 weeks after injection, respectively. To study proliferation, 24 hours was chosen, as this is sufficient for a newborn cell to complete at least one cell cycle.

**Acute stress protocol**

To study whether acute stress induced lasting reductions in newborn cell proliferation, rats were stressed for one day, by cold immobilization (1 h at 4°C) in the morning and by forced swimming (30 min at 25°C) in the afternoon. Two stressors were selected, since in the chronic paradigm animals were also subjected to two stressors per day. One part of the acutely stressed rats (group I; n = 6) received BrdU on the morning prior to stress exposure and was perfused 24 hours later (injection scheme in Fig. 1A). The other group (group II; n = 6) was injected with BrdU the day following their subjection to stress and then further allowed to survive for another 24 hours. This latter group was included to answer the question whether the acute stress effect is lasting, and also served as a control for the last day of the chronic stress protocol, concerning the timing of the BrdU injection.
Chronic stress protocol

Rats were subjected to chronic unpredictable stress according to protocols described earlier (Herman et al., 1995). This paradigm was selected since it was shown to be associated with corticosterone hyper secretion (Herman et al., 1995; Paskitti et al., 2000) and CA3 dendritic atrophy (Magarinos et al., 1996). Briefly, rats were exposed to different stressors twice daily for 21 days as follows: day 1: cold immobilization for 1 h at 4°C; forced swim for 30 min at 25°C; day 2: immobilization for 1 h; crowding for 24 h (overnight); day 3: forced cold swim stress for 5 min at 10-15°C; isolation for 24 h (overnight); day 4: immobilization for 1 h; vibration for 1 h; day 5: forced swim stress for 30 min at 25°C; cold immobilization for 1 h at 4°C; day 6: forced cold swim stress for 5 min at 10-15°C; crowding for 24 h (overnight); day 7: vibration for 1 h; isolation for 24 h (overnight). This schedule was repeated twice to a total of 21 days. To exclude effects of handling of the stressed rats, control rats were handled twice daily. On the other hand, certain criteria were set for excluding animals from the study based on weight loss, or the possible occurrence of wounds. No animals were excluded from the study.

BrdU injection was performed either one day before the beginning (3 weeks S), or one day after the end (24 hours S) of the 21-days of chronic stress, recovery or handling period (injection scheme in Fig. 1B).

Figure 1A:

Figure 1B:
Corticosterone levels

In order to determine basal corticosterone levels, blood samples were drawn in the morning before perfusion of the rats. Blood was centrifuged for 20 min at 5000 rpm (1900 g) at +4°C and the plasma stored at -20°C until corticosterone concentrations were determined using radio immunoassay (ICN Biochemicals Inc.).

Brain tissue preparation

Animals were deeply anaesthetised in the morning by i.p. injection of pentobarbital sodium salt (Nembutal 1 ml/kg body weight; A.U.V., Cuijk, The Netherlands) and then perfused transcardially with 0.9% physiological saline followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. After in situ postfixation overnight at 4°C, the brains were taken out and the two hemispheres separated by a midline cut. The left hemisphere was then equilibrated in 30% sucrose, frozen and sectioned in a coronal plane at 40μm thickness using a sliding microtome. Sections were stored at -20°C in 2% Dimethylsulphateoxide (DMSO), 20% glycerol, 0.05M Tris buffer pH 7.6 until needed.

BrdU visualization

BrdU-labeled nuclei were visualized as described before (Heine et al., 2003). Briefly, after blocking endogenous peroxidase and denaturing DNA, free-floating sections were incubated with the primary antibody mouse α-BrdU (Roche Diagnostics, Netherlands, 1:3000) diluted in phosphate buffer (PB) / 0.1% bovine serum albumin (BSA) / 0.3% Triton X-100 / 1% goat serum for 1 hr at RT and then overnight at 4°C. With intermittent rinses in PB, sections were subsequently incubated with biotinylated sheep α-mouse IgG (Amersham Life Sciences, Den Bosch, Netherlands, 1:200) for 2 hrs and Elite Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories, Brunschwig Chemie, Amsterdam, Netherlands, 1:1000) for 2 hrs. Color development was performed with diaminobenzidine (0.50 DAB mg/ml Tris/HCL, 0.01% H₂O₂) for 30 min, after which sections were washed, mounted, dehydrated, passed through xylene and coverslipped with Entallan (Merck).

Ki-67 immunoperoxidase

Since BrdU incorporation in newborn brain cells may be influenced by peripheral factors, like liver clearance and brain penetration, we also included the endogenous proliferation marker Ki-67. The Ki-67 antigen is a 345 to 395 kDa non-histone protein
complex present only in proliferating cells during G₁, S, G₂ and M, but not the G₀ phase of the cell cycle (Gerdes et al., 1984; Endl & Gerdes, 2000). Ki-67 numbers were indeed shown to be highly comparable to BrdU counts after short survival times (Kee et al., 2002). Moreover, Ki-67 is a well-accepted proliferation marker in tumor biology (Gerdes et al., 1991).

To detect Ki-67 positive nuclei, free-floating sections were mounted onto Plus glass slides (Menzel) and dried overnight at 37°C. Following a short rinse in 0.1M Tris buffered saline pH 7.6 (TBS), sections were placed in plastic jars filled with citrate buffer (0.01 M, pH 6.0) and placed in a microwave oven. For standardization purposes, always two filled jars were used, irrespective of the number of sections, which rotated in the middle of a domestic MW oven (Samsung M 6235, 800 W). Microwave treatment took 15 min in total, starting at 800 W for 5 min, subsequently lowering to 400 W and 260 W to prevent extensive boiling / tissue damage. After 30 min cooling at room temperature, endogenous peroxidase activity was blocked by 15 min 1.5% peroxide treatment in TBS.

After several rinses in TBS, 2% milk powder (Elk, Campina Melkunie, Eindhoven, The Netherlands) solution was applied for 30 min to reduce nonspecific binding. Sections were then incubated with the primary antibody polyclonal rabbit α-Ki-67 (Novocastra, New Castle, UK, 1:2000) diluted in 0.25% gelatin / 0.5% Triton X-100 (Supermix) for 1 hr at RT and then overnight at 4°C. With intermittent rinses in TBS, sections were incubated with biotinylated sheep α-rabbit IgG (Amersham Life Sciences, Den Bosch, Netherlands, 1:200) in Supermix for 1.5 hrs and amplified with ABC (1:800) in TBS / BSA 1% for 2 hrs. The ABC signal was further amplified with biotinylated tyramide (1:500, produced and kindly provided by Dr I. Huitinga, Neth. Inst. for Brain Research, Amsterdam) and 0.01% peroxide in TBS for 30 min followed by another 1.5 hr incubation with ABC (1:1000). Chromogen development was performed with diaminobenzidine (0.50 DAB mg/ml Tris/HCL, 0.01% H₂O₂) for 6-10 min after which sections were washed, dehydrated, passed through xylene and coverslipped with Entallan.

**TUNEL**

To detect cells undergoing apoptosis, terminal transferase mediated dUTP nick-end-labeling (TUNEL) was performed as described in detail earlier (Lucassen et al., 1997; Lucassen et al., 2000; Heine et al., 2003) with minor modifications. Free-floating sections were mounted onto Plus glass slides, dried overnight at 37°C and rinsed with 0.01 M PBS (KH₂PO₄, Na₂HPO₄.H₂O) pH 7.2. Sections were then microwave pretreated in sodium citrate buffer (0.1 M, pH 6.0). After that, the jars were allowed to cool for 10 min before pre-incipubation with Proteinase K buffer (10 mM Tris/HCL, 2.6 mM CaCl₂, pH 7.6) for 10
min and then incubated with 20 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) in PK buffer for 15 min at room temperature. After a brief rinse in double distilled water (DDW) and wash in PBS, sections were pre-incubated with terminal transferase (TdT) buffer (0.2 M sodium cacodylate, 0.025 M Tris/HCl and 0.25 mg/ml BSA, pH 6.6) for 10 min and incubated for 60 min at 37°C with a reaction mixture containing: 0.1 μl TdT, 0.5 μl biotin-16-dUTP (Boehringer Mannheim, Almere, Netherlands) per 100 μl reaction mixture and 5% cobalt chloride (25 mM). Incorporation of labeled oligonucleotides was ended by briefly rinsing in DDW. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in PBS for 15 min after washing with PBS. Following another wash in PBS, sections were pre-incubated with PBS / 1% BSA for 15 min and incubated with ABC (1:1000) in PBS / 1% BSA for 2 hours. Chromagen development was done with 0.50 DAB mg/ml Tris/HCL, 0.01% H₂O₂ for 10 min. Sections were lightly counterstained with cresyl violet.

**BrdU / NeuN immunofluorescence**

Co-localization of the neuronal marker NeuN with BrdU immunoreactivity was examined in the subgranular zone (SGZ) and granular cell layer (GCL), 3 weeks after BrdU injection. For DNA denaturalization, sections were pretreated as described above for BrdU. Afterwards, sections were rinsed in 0.05 M TBS and incubated in TBS / 1% BSA / 0.1% Triton X-100 / 3% goat serum (TBS++) for 1 hr at room temperature and then for 48 hrs at 4°C with the primary antibodies: rat anti-BrdU (1:3000, Accurate Chemicals, Westbury, NY) and mouse anti-NeuN (1:5000, Chemicon, Harrow, UK) diluted in TBS++. After several rinses in TBS and incubation in TBS++ for 30 min, the first antibodies were subsequently detected with Alexa Fluor 546 (1:500) and Alexa Fluor 488 (1:1000, Molecular Probes, Leiden, The Netherlands), respectively and embedded in Vectashield (Vector Laboratories, Brunschvig Chemie, Amsterdam, The Netherlands).

**Quantification and stereology**

Serial sections (40 μm, every 12th section) of one hemisphere were taken for stereological quantification of volumes of the different hippocampal subregions, dentate granule cell number, as well as for TUNEL-, BrdU- and Ki-67-positive cell numbers. To study distribution and also migration / survival of newborn cells, their numbers were assessed per hippocampal subregion, i.e., the hilus, SGZ, GCL as well as molecular layer in a stereological approach over the entire rostro-caudal extent of the hippocampus. The subgranular zone was defined as a two-cell layer thick zone along the inner border of the GCL and the hilus.
Cresyl violet stained serial sections were used to determine the total volume of the granule cell body layer, the hilar region, the CA3 cell body area and part of the stratum radiatum / lucidum of the CA3 according to anatomical criteria defined previously (Lavenex et al., 2000), and calculated according to Cavalieri's direct estimator (Gundersen & Jensen, 1987). Individual sections were viewed on a video monitor connected to a Zeiss Axiophot microscope (5× NA 0.15 objective) at a final magnification of 125×. The hilar region (hilus) was defined by drawing straight lines from the tip of the two GCL blades towards the tip of the CA3 layer. Part of the stratum radiatum / lucidum of the CA3 (Str Rad /Luc) was then delineated by placing an extra line between the tip of the inner GCL blade and the CA3/CA2 border (see also Fig. 6A). The different regions were measured on a Macintosh computer for the full rostral-to-caudal series, using the public domain program Object-Image (an extended version of NIH Image, developed at the U.S. National Institutes of Health and at the University of Amsterdam; available from http://simon.bio.uva.nl). Total volumes were calculated from \( V = \sum A \times T \), where \( \sum A \) is the sum of area measurements and \( T \) is the intersection distance 480μm.

The same sections were used to determine the numerical density of neurons (Nv, neurons per cubic millimeter) in the GCL, using the optical disector method (West et al., 1991). Object-Image software was used to randomly place a rectangular counting frame over the GCL on the reference section. Individual neurons were visualized using a Zeiss Axiophot microscope (100× NA 1.30 oil objective) and counted with the disector if their nuclear profile was present in the reference section but not in the look-up section and if they were positioned within the counting frame or intersected by its inclusion edges (i.e., the top and the right edge). Per hippocampus more than 100 dissectors were used to calculate the numerical density (Nv) of neurons from \( N_v = \frac{\sum Q \times V_{\text{dis}}}{\sum V_{\text{dis}}} \), where \( \sum Q \) is the sum of the neurons counted in all dissectors and \( \sum V_{\text{dis}} \) is the sum of the disector volumes. One disector volume is 23040 μm³, as calculated from \( V_{\text{dis}} = a_{\text{dis}} \times h \), where \( a_{\text{dis}} \) is the area of the square counting frame, measuring 24 μm on a side, and \( h \) the disector height of 40 μm. The total number of neurons in the GCL of one hemisphere was calculated by multiplying the \( N_v \) with the total volume of the GCL.

BrdU/NeuN double immunostained sections (40 μm thick, every 24th section) were evaluated using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal laser-scanning device equipped with a Plan-Neofluar 100× / 1.3-oil lens. Co-localization of NeuN with BrdU was determined after visual inspection of the XY, YZ and XZ views. To assess whether the extent of neuronal differentiation of the newborn cells was influenced by stress exposure, the proportion of co-localization was calculated in the control (n = 5).
and chronically stressed (n = 5) rats, based on 50 BrdU-positive cells randomly selected over the (approximately 5) serial sections.

Statistics

Statistical analysis was performed using an unpaired Student’s t-test with a two-tailed p-value. Differences are considered significant, when the two-tailed P-value < 0.05. When standard deviations were not equally distributed between the two groups, a non-parametrical Mann-Whitney-U-test was applied to the data.

Results

Acute Stress

The lasting effect of acute stress on new cell proliferation was tested in two groups (I and II), which differed in the timing of BrdU injection (injection scheme in Fig. 1A; results are presented in Fig. 2). Group I received BrdU on the morning prior to the stress exposure, and survived 24 hours afterwards. This group revealed significantly less BrdU-positive cells in the entire dentate gyrus (Fig. 2: h + SGZ + GCL; p = 0.02 as compared to controls). This effect was due to fewer proliferating cells in all DG sub-regions, but only reached significance in the GCL (p = 0.01). When rats (group II) were injected with BrdU the day after the acute stress exposure and perfused 24 hours thereafter, the number of proliferating cell had already returned to basal levels and was not significantly different anymore (h + SGZ + GCL; p = 0.24). This rapid normalization was confirmed by Ki-67 immunocytochemistry on group I. The number of proliferating Ki-67-identified cells in the entire DG one day after acute stress was not different from controls (h + SGZ + GCL: Acute stress group I: 11200 ± 596 versus Control: 11819 ± 376; p = 0.23).

Figure 2A:

Figure 2: Proliferation of newborn cells in the adult hippocampus is decreased by acute stress, and recovered to normal levels after one day. Animal with BrdU injection on the morning of the day of stress exposure (Ac Stress, group I) showed a significant decrease in the amount of newborn cells in the GCL (p = 0.01) and total dentate gyrus (h + SGZ + GCL) (p = 0.02). Group II reflects BrdU injection on the morning after the stressors were
applied (1d Recovery) and was studied 24 hours thereafter. (A): Numbers of proliferating cells are expressed as the estimated mean total number (± SEM) of BrdU- (24 hours survival) positive cells per hippocampal region in the control and acutely stressed rats (n = 6 for each group). (B): Typical example of BrdU-immunocytochemistry in a control (B1) and acutely stressed (B2) rat.

These animals were also studied for the effect of stress on apoptotic cell death (Fig. 3, see Appendix for Figure 3B). TUNEL-positive apoptotic cells were observed in all DG sub-regions and showed a significant increase in the hilus (p = 0.007), the SGZ (p = 0.003), the GCL (p = 0.004) and consequently also over the whole dentate gyrus (p = 0.002), but only in rats examined the morning after they were subjected to stress (group 1). The effects on apoptosis were absent in rats that were allowed to recover for one more day (h + SGZ + GCL: group II; p = 0.33).

Figure 3: Apoptotic cell death in the hippocampal dentate gyrus increased significantly after 1-day of acute stress exposure, in the main DG sub-regions (group I), but normalized on the day after (group II). (A): Numbers of apoptotic cells are expressed as the estimated mean total number (± SEM) of TUNEL-positive cells per hippocampal region (n = 6 for each group). Asterisk indicates a statistically significant difference with the control group (p < 0.01). (B, see Appendix):
To measure corticosterone levels, blood samples were drawn in the morning (between 9 and 12 am), just before the rats were perfused. Elevated Cort levels in group I (5.11 ± 1.96 μg / dl; n = 6) were not significantly different ($p = 0.25$) from controls (2.31 ± 0.85 μg / dl; n = 5); neither were the basal cort levels in group II (1.91 ± 0.42 μg / dl; n = 5).

**Chronic Stress**

During the chronic stress experiment, changes in body weight of rats in various treatment groups were monitored (Fig. 4). The growth rate of the chronically stressed rats was impaired, while that of the recovering animals had normalized 3 weeks after stress.

![Figure 4](image)

**Figure 4:** The growth rate was impaired during chronic stress. Animals of the recovery group started off with a decreased body weight at the age of 7 weeks (end of their stress period), but their weight gain normalized during the additional 3 weeks of survival ($n = 10$ for each group).

As indicator for HPA-axis activity, reliable Cort levels could not be measured due to perfusion of the animals. Our current animals were, however, part of a larger group used for electrophysiological analysis that experienced the exact same paradigm.

In this larger group of pooled control ($n = 21$) and chronically stressed ($n = 31$) animals, significant increases in Cort levels were found in the latter (Chronic stress: $4.50 ± 1.08$ versus Control: $1.56 ± 0.30$ microgram / dl; $p < 0.04$).

The total volumes of various hippocampal sub-regions were stereologically determined (Fig. 5), as well as the total number of granule cells in the dentate gyrus. The CA3, here defined as the surface area mainly occupied by the cell bodies of the CA3 pyramidal neurons, was significantly reduced in volume after chronic stress ($p < 0.01$, Mann Whitney test). This reduction in surface area had not yet returned to control values after three weeks of recovery ($p = 0.03$). Other sub-regions showed no differences in surface areas. Also, the granule cell layer did not differ in volume (Fig. 5) nor cell number.
(Chronic Stress: 652290 ± 18675 versus Control: 693413 ± 33871) relative to controls.

**Figure 5A:**

![Diagram of hippocampal subregions](image)

**Figure 5B:**

![Bar graph showing volume comparison](image)

Figures 5: Three weeks of chronic stress induced a reduction in the CA3 surface area (p < 0.01, Mann Whitney test), which failed to recover after an additional 3 weeks of survival (p < 0.03). (A): Schematic representation of the measured hippocampal subareas adopted after (Lavenex et al., 2000). (B): Mean estimated volumes (±SEM) of the main hippocampal subregions: granular cell layer (GCL), hilar region (hilus), CA3 cell body area (CA3) and part of the stratum radiatum / lucidum of the CA3 (Str Rad/Luc), in 10-weeks old Wistar rats (n = 10 for each group).

Numbers of proliferating cells were measured by calculating total amounts of BrdU- (24 hours survival) and Ki-67-positive cells (Fig. 6) per hippocampal region, and integrated for the entire hemisphere. Most of the proliferating Ki-67-positive cells were observed in the SGZ, a considerable amount in the hilar region and very little in the GCL and molecular layer (Fig. 6). Three weeks of chronic stress significantly decreased the numbers of newborn cells, in the hilus (p = 0.0002), SGZ (p < 0.0001) and the total DG (h + SGZ + GCL; p < 0.0001) as compared to controls. After an additional three weeks of recovery, levels were increased significantly as compared to chronically stressed rats (SGZ: p < 0.04; h + SGZ + GCL: p = 0.03), but did not recover completely to control levels, and the numbers in the recovered animals were still significantly lower than in controls (SGZ: p < 0.02; h + SGZ + GCL: p < 0.03).
Part I: Stress

Figure 6: Ki-67 immunocytochemistry reveals a significantly decreased proliferation rate in the hilus (p = 0.0002), SGZ (p < 0.0001) and in the total DG (hilus + SGZ + GCL) (p < 0.0001) of chronically stressed rats. After an additional three weeks of survival, levels again increased significantly compared to the chronically stressed rats (SGZ: p < 0.04; h + SGZ + GCL: p = 0.03), but did not recover completely to control levels, and numbers of the recovered animals were still significantly lower (SGZ: p < 0.02; h + SGZ + GCL: p < 0.03). (A): Proliferating cell numbers are expressed as the estimated mean total number (± SEM) of Ki-67 positive cells per hippocampal region of the control (n = 10), chronically stressed (n = 11) and recovered 10-weeks-old rats (n = 6). (B): Typical example of a Ki-67 positive cells in the hippocampal DG / SGZ region of a control (B1) and chronic stress (B2) animal.

BrdU-positive cells (24 hours survival) showed a similar distribution over hippocampal areas, as found with Ki-67. With this method, fewer newborn cells were seen in the chronically stressed rats in the hilus and SGZ, but this failed to reach significance, possibly due to the small group size (Chronic Stress, n = 4: 3951 ± 365 <-> Control, n = 3: 4888 ± 326 BrdU-positive cells in the total DG). The number of Ki-67-positive cells is larger (~1.5-1.6 fold) than the number of BrdU-positive cells after 24 hrs of survival. Thus both markers showed a reduction in newborn cell proliferation in the hilus and SGZ, with the Ki-67 number reaching statistical significance. The data indicate that new cell
proliferation is decreased in the rat dentate gyrus after chronic unpredictable stress; an additional survival of three weeks already yields partial recovery already.

To test if chronic stress influences the survival of newborn cells, rats were injected with BrdU one day before chronic stress started and perfused the morning after the last stressor was applied (Fig. 7), 3 weeks later. Most BrdU-positive cells were found in the GCL, indicating that surviving cells had migrated from their place of birth into this region. Also the morphology of older BrdU-positive cells was clearly different, as they were considerably larger, with the BrdU signal more dispersed over the nucleus. No change in the amounts of BrdU-positive cell in any sub-region or in the total dentate gyrus (h + SGZ + GCL) was observed.

Figure 7A:

![Graph showing the number of newborn cells after three weeks survival is not significantly different between chronically stressed and recovered rats.](image)

Figure 7B:

![Micrograph showing BrdU-immunohistochemistry, 3 weeks survival, in a 10-week old rat.](image)

To study the neuronal phenotype of the newborn cells 3 weeks after BrdU injection, sections were double-labelled for BrdU and the neuron-specific marker NeuN. Fluorescent images were inspected in the orthogonal planes X, Y and Z to verify double labelling throughout the extent of the cells.
The percentage of double-labelled cells was not changed after the chronic stress period (Chronic Stress: 50 ± 6% <-> Control: 52 ± 4% BrdU-NeuN double-positive cells; \( p = 0.81 \); \( n = 5 \) rats in both groups). Therefore, we conclude that although proliferation is profoundly affected, chronic unpredictable stress has no effect on migration, survival or neuronal differentiation of the newborn cells.

Apoptotic cell death was studied in all animals. TUNEL-positive cells were quantified in the entire DG after chronic stress and after 1-day or 3-weeks period of recovery (Fig. 9). In control rats, most dying cells were observed in the SGZ, and less so in the other regions (Fig. 9). Similar to the results after acute stress (Fig. 2), the number of dying cells was significantly increased after chronic stress in the GCL region (\( p = 0.002 \)). This did not normalize after 1 day (\( p = 0.001 \)) or even 3 weeks (\( p = 0.004 \), Mann Whitney test) of recovery (Fig. 9). An opposite effect was observed in the SGZ, where a significant and strong decrease in the numbers of TUNEL-positive cells was measured (\( p < 0.0001 \)). This decline did not recover within one day (\( p = 0.002 \), Mann Whitney test), but returned to basal levels after 3 weeks. So chronic stress differentially affects apoptosis in the hippocampus. Overall, apoptosis decreased in the whole dentate gyrus (h + SGZ + GCL; \( p = 0.004 \), Mann Whitney test), which lasted for at least one day (\( p = 0.002 \)), but normalized after 3 weeks.

**Figure 9:** Chronic stress differentially affected apoptotic cell death in regions of the hippocampus. In the SGZ, the number of TUNEL-positive cells was significantly decreased the day after chronic stress (\( p < 0.0001 \)). This decline did not normalize within one day of recovery after the chronic stress exposure (\( p = 0.002 \), Mann Whitney test), but did return to basal levels after 3 weeks. The amount of apoptotic cells was significantly increased in the GCL (\( p = 0.002 \)), but did not normalize after 1 day (\( p = 0.001 \)) or even 3 weeks (\( p = 0.004 \). Mann Whitney test) of recovery. Overall, apoptosis decreased in the whole dentate gyrus (h + SGZ + GCL; \( p = 0.004 \), Mann Whitney test), which lasted for at least one day (\( p = 0.002 \)), but normalized after 3 weeks. (A): Numbers of apoptotic cells are expressed as the estimated mean total number (± SEM) of TUNEL-positive cells per hippocampal region in the control (\( n = 6 \)), chronically stressed rats (\( n = 5 \)), and the 1-day (\( n = 6 \)) and 3-weeks (\( n = 6 \)) recovery groups.
Discussion

In the present study, we used a well-established mixed stress paradigm to show that acute as well as chronic stress decrease new cell proliferation in the adult rat DG. The reduced proliferation rate found after acute stress was already completely normalized after one day. Interestingly, suppression of proliferation did last for at least one day after chronic stress, but was nevertheless partially recovered after an additional recovery of 3 weeks. Migration, survival rate or the percentage of newborn cells that differentiated into a neuronal phenotype, did not change during 3 weeks of chronic stress. Cell death on the other hand, reversibly increased after acute stress in the main DG sub-areas. After 3 weeks of chronic stress, overall cell death was decreased in the DG, and normalized by 3 weeks of recovery. Together, this suggests that the impact of acute stress is short lasting; however, chronic stress has longer-lasting effects on the main structural-dynamic hippocampal parameters, which are mostly normalized after 3 weeks of recovery.

Several stress paradigms have already been shown to decrease granule cell proliferation in adult rat, tree shrew and marmoset (Gould et al., 1997; Gould et al., 1998; Tanapat et al., 1998; Tanapat et al., 2001). Importantly, most of these studies involved acute and/or psychosocial stressors, e.g. the dominant-subordinate model in tree shrews (Gould et al., 1997; Fuchs et al., 2001), the resident-intruder stress in rat and monkey (Gould et al., 1998; Czeh et al., 2002), or exposure to predator odor in rat (Tanapat et al., 1998). A large reduction (76%) in proliferation was found after acute psychosocial stress in the tree shrew (Gould et al., 1997), while chronic stress exposure in this species, that was notably accompanied by persistently elevated cortisol levels, already revealed a much milder reduction (33%) (Czeh et al., 2001). As stated also by others (Czeh et al., 2002), proliferation rate might habituate following chronic stress and display a reduced sensitivity to HPA hormones. In line with this, stressor repetition can cause habituation of neuronal and non-neural stress parameters after chronic stress (Magarinos & McEwen, 1995).

Habituation, though, may depend on the type of stressors applied. A very recent study showed that stressors applied acutely, did not change DG proliferation, whereas 3 weeks of physical stress (restraint for 6 hrs per day) did suppress proliferation; 6 weeks even further decreased neurogenesis (Pham et al., 2003). In our paradigm of chronic unpredictable stress we also found no signs of habituation: Acute stress as well as chronic stress both reduced DG cell proliferation. We selected this paradigm for two reasons. First, with this paradigm rats show impaired weight gain, increased adrenal and decreased
thymus weight (Alfarez et al., 2003; Karst & Joels, 2003; van Riel et al., 2003), as well as somewhat elevated basal corticosterone levels, which are all classical features of chronic stress and HPA-axis hyperactivity. Although MR and GR-mRNA expression (van Riel et al., 2003) nor GR binding was altered in the model (Herman & Spencer, 1998; Paskitti et al., 2000), it does produce CA3 volume reduction, consistent with the stress effects on dendritic structure in this region (Magarinos et al., 1996; Galea et al., 1997); and it exerts specific changes in electrophysiological properties of DG granule cells, the DG network and CA1 neurons (Alfarez et al., 2003; Karst & Joels, 2003; van Riel et al., 2003).

Secondly, the selected paradigm consists of an unpredictable mixture of (psycho)social and physical stressors (Herman et al., 1995). This not only reduces the risk of adaptation, but also better mimics the variability of stressors encountered in daily life, especially when compared to chronic restraint.

When comparing our present data on proliferation with other studies, the number and timing of BrdU injections (e.g., before or after the applied stressor) are important methodological considerations that should be taken into account; particularly when changes in proliferation are only transiently present. Earlier papers on neurogenesis, running and learning e.g., that shared a comparable experimental design, but differed surprisingly in their main results, illustrate this (Gould et al., 1999; van Praag et al., 1999); was elegantly discussed separately (Greenough et al., 1999). Moreover, also our own acute stress experiments point out the importance of the timing of the BrdU injections. BrdU administration prior to acute stressor application (group I) demonstrated impaired proliferation, while rats that received BrdU the day after being subjected to the stress paradigm (group II), did not show impaired proliferation anymore. This was confirmed by Ki-67 immunocytochemistry, an independent marker for adult proliferation, which identifies all proliferating cells engaged in the cell cycle (Kee et al., 2002).

A similar consideration regarding time after stress holds when comparing data on apoptotic cell death. In our study, cell death increased in the main sub-regions of the DG when studied the day after a one-day stress paradigm; yet, the increased numbers of TUNEL-positive cells had already normalized one day later. This rapid normalization may explain the discrepancy with a study by Hassan (1996), who did not find increases in apoptosis 24 hrs after a single Cort injection (N.B. not stress), in any of the different age groups studied. Cell death in these animals may have already recovered 24 hours after Cort injection. However, other stress related factors in addition to elevated Cort levels could also have been responsible for this discrepancy.

Because both apoptosis was increased and proliferation was decreased after
acute stress, either a rapid and short-lasting upregulation of turnover in the entire DG, or a compensatory cell death counteracting new cell birth takes place. This latter possibility is consistent with the close association of the two processes in the adult DG (Kuhn et al., 2001). Surprisingly, after chronic exposure to stress a rather heterogeneous picture of cell death appeared: Much lower numbers of dying cells are found in the SGZ, increased numbers in the GCL, but overall less cell death in the entire DG compared to controls. This impaired cell death was still present after one-day recovery but had normalized after 3 weeks. Similarly, in adult tree shrews, 3 weeks of psychosocial stress also decreased apoptosis in the hilar region of the dentate gyrus (Lucassen et al., 2001a), suggesting this effect may not be limited to rats alone.

Several explanations can be given for the heterogeneity in apoptotic changes. In view of the trisynaptic hippocampal circuit, apoptosis induced in e.g. CA3, could in theory have contributed to apoptosis in other sub-regions at later time-points, through anterograde or retrograde projections. However, we did not find such increases in apoptosis in the CA3 after acute stress. Furthermore, as indicated above, large numbers of cells may have died already before the end of the 3 weeks stress period, resulting in lowered apoptotic numbers in a given region, as fewer cells are still available to engage in apoptosis. Clearly, there is less apoptosis in the SGZ, which could be explained by a decreased presence of young proliferating cells, as it is known that around 50 percent of the newborn cells dies within the first 2 weeks after birth (Cameron et al., 1993b; Gould et al., 1991b; Heine et al., 2003). Moreover, we found that the number of newborn neurons that do survive and differentiate into a neuronal phenotype does not change by chronic stress. The 50% of new neurons in both groups is well in line with another report on their phenotypic analysis (Kempermann et al., 1998). Therefore a higher percentage of the stress-induced reduced numbers of proliferating cells is expected to survive. This implies that less apoptosis in the SGZ could be ascribed to more survival of newborn cells in this region. Since TUNEL does not identify the phenotype of the dying cell, it remains to be proven whether stress preferentially increased death of newborn, rather than residing, adult granular cells, or possibly even glia or interneurons. Differential susceptibility to cell death may depend on the age of individual cells or on the extent and type of their established connections and synaptic input (Schlessinger et al., 1975; Gould et al., 1991b; Gould et al., 1994). In view of this heterogenous nature of the DG, it is most likely that distinct types of cells in the GCL react differently to acute and chronic stress, a phenomenon also seen after ADX or NMDA receptor blockade (Cameron & Gould, 1996; Hassan et al., 1996). This awaits, however, further research.
Which factor mediates the effects of acute and chronic stress on proliferation and cell death in the adult DG? In general, the stress-associated rise in glucocorticoid levels is believed to be crucial in mediating the effects of stress on adult cytogenesis (Gould et al., 1991c; Gould et al., 1994; Gould et al., 1998). The DG contains high concentrations of corticosteroid receptors and is indeed highly sensitive to changes in corticosteroid levels with regards to the regulation of cell birth and cell death. Rats treated with glucocorticoids or mineralocorticoids during development, showed substantially diminished cell death in the GCL, but increased numbers of dying cells in the hilar region (Gould et al., 1991c). Furthermore, removal of corticosteroids by adrenalectomy (ADX) in adult rats stimulates the production of new granule cells (Cameron & McKay, 1999; Montaron et al., 1999) but produces at the same time massive apoptosis in the DG (Sloviter et al., 1989; Gould et al., 1991a), further demonstrating a close association between cell birth and cell death in this region.

Indeed, in our acute stress group (I), significant effects on cytogenesis and apoptosis were paralleled by a transient rise in corticosterone levels, which seemed to be still lingering on when animals were sacrificed following the one-day stress paradigm; yet, one day after the stress paradigm levels were back to control (group II). This is consistent with a recent study in rats demonstrating that persistently elevated corticosterone levels, rather than endogenous circadian fluctuations in this hormone, are crucial for the reductions in cell proliferation in the DG (Ambrogini et al., 2002). How exactly Cort exerts its effect on the dynamic turnover is not known. So far adrenal steroid receptor expression was not found on granule cell precursors (Cameron et al., 1993a), suggesting that corticosteroids act via different pathways, possibly through increased glutamate levels, or via NMDA receptor mediated actions (Cameron et al., 1995; Reagan & McEwen, 1997), while also the vasculature may be implicated in hippocampal neurogenesis (Palmer et al., 2000). Interestingly, rats subjected to the chronic unpredictable stress paradigm showed significantly increased Cort levels, on the day after the last stressor was applied. This in contrast to levels found after acute stress exposure. What is the impact of altered cell birth and death after chronic stress on the permanent structural DG parameters i.e. volume and cell number? Stressful experiences are commonly considered to have a negative impact on brain function and structure. Although the initial, acute responses to stress are generally considered to be an adaptive mechanism, chronic and uncontrollable stress can cause deterioration of learning and memory (Dellu et al., 1992; Luine et al., 1994; Conrad et al., 1996) and induce an enhanced vulnerability to damage. Previous studies have suggested that chronic stress and/or elevated levels of Cort could even induce cell loss, mainly
in the pyramidal layers (Uno et al., 1989; Jacobson & Sapolsky, 1991; McEwen et al., 1993; Landfield & Eldridge, 1994). Considerable controversy exists in the literature as to whether long-lasting stress exposure and/or elevated glucocorticoids induce transient structural changes, or result in irreversible neurodegeneration (Vollmann-Honsdorf et al., 1997; Sousa et al., 1998; Leverenz et al., 1999; Lucassen & De Kloet, 2001; Lucassen et al., 2001a). Yet, several alterations are consistently observed, involving mainly dendritic atrophy of CA3 pyramidal neurons (Woolley et al., 1990; Magarinos & McEwen, 1995; Conrad et al., 1999), alterations in presynaptic mossy fiber terminals (Magarinos et al., 1997; Sousa et al., 2000) and atrophy of granule and CA1 cells (Woolley et al., 1990; Pham et al., 2003).

Similarly, in the present study we also found a decrease in CA3 volume, confirming the effectiveness of our stress paradigm. The decrease in CA3 volume remained present even after the 3 additional weeks of recovery. This suggests that CA3 atrophy may require more time to normalize than the dynamics of the DG cells projecting to it. In the DG we did not find changes in volume or cell number, which is consistent with the absence of any alterations in the number of surviving newborn cells or neurogenesis, we found. Bodnoff et al. (1995) failed to find any hippocampal loss in middle-aged rats whose spatial learning abilities were disrupted by Cort treatment. Sousa et al. (1998) failed to find cell loss in any hippocampal division after chronic unpredictable stress or chronic cort treatment, even though they measured persistently high corticosterone levels and used modern stereological methods for quantification. In aging Wistar rats, neurogenesis or DG cell number or volume could not be related to basal GC levels, or to stress responsiveness either (Heine et al., 2003).

Taken together, this suggests that the impact of chronic stress or enhanced GC levels on the permanent structural DG parameters volume and cell number is rather modest, and may require prolonged and severe exposure (e.g. 6 weeks restraint, for 6 hours per day), before relatively small reductions (i.e. 5%) become detectable (Pham et al., 2003). It seems that a.o. by diminishing the incidence of apoptosis, survival of new cells can be enhanced to keep production of new neurons constant (neurogenesis). So it remains questionable what the relative contribution of DG changes is to volume reductions of the hippocampus as a whole, as found in depressed patients. This could to a large extend be subscribed to the CA3 area. This does not necessarily imply that the functional implications of changes found in the DG are also modest. The temporary suppression in turnover indicates that following chronic stress the overall identity of DG cells and thus their connectivity may be different from control situations.
As to the chronic nature of the stress, it obviously remains difficult to compare a human disorder like major depression with a rat model of chronic stress. However, the present chronic stress paradigm does induce alterations in HPA parameters and lasting structural volume reductions (Herman et al., 1995). Comparable durations of stress are also applied in other models of e.g. psychosocial stress (Fuchs et al., 2001; Lucassen et al., 2001a). When animals were allowed to recovery for 3 weeks, i.e. the same period required to induce these structural changes, most parameters were already normalized. Stress exposure for longer periods may have different effects and more time is likely to be needed for recovery.

Although changes in cell turnover after chronic stress are much more prolonged than after an acute stress, partial recovery can already be seen within 3 weeks of recovery from chronic stress. This is reminiscent of hippocampal volume reductions seen in depressed patients (Sheline, 1996), which are often reversible following antidepressant medication or therapy. Indeed, these changes do not seem to represent permanent hippocampal damage, as no indications for neuropathological alterations or major cell loss could be found in the hippocampus of major depressed individuals (Lucassen et al., 2001b; Muller et al., 2001). Chronic stress has been causally implicated in major depression and indeed various structural and endocrinological markers, like increased adrenal volume, large percentages of dexamethasone non-suppressors and an hypothalamic hyperdrive, all indicate enhanced HPA activity in a large percentages of these patients ([Raadsheer, 1995 #107; Holsboer & Barden, 1996; Lucassen et al., 2001b]). Importantly, many antidepressant drugs interfere with neurogenesis or apoptosis (Madsen et al., 2000; Malberg et al., 2000; Manji et al., 2001; D'Sa & Duman, 2002; Kempermann, 2002; Lucassen et al., 2003), suggesting that their therapeutic effects may at least in part occur through modulation of new cell birth or death in the DG, that in turn, may influence cell viability and cell number. It may be of interest to examine in future whether administration of antidepressants and antiglucocorticoids can prevent the changes in proliferation and cell death brought on by chronic unpredictable stress in rat.
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