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

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Publication date
2004

Citation for published version (APA):

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Download date:22 Sep 2023
Chapter 5

Chronic Stress Reduces Vascular-Associated Adult Proliferation, VEGF and Flk-1 Protein Expression in the Rat Dentate Gyrus

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submitted
Abstract

Recent evidence has shown that cell proliferation in the adult hippocampal dentate gyrus occurs in tight clusters located near the vasculature. Also, changes in neurogenesis often appear parallel to changes in angiogenesis. Moreover, both these processes share similar modulating factors, like vascular endothelial growth factor (VEGF) and its receptor Flk-1. In an earlier study we found that chronic stress decreased new cell proliferation in the adult dentate gyrus. We here questioned whether these effects of chronic stress are mediated through the vasculature and whether they involve an angiogenic-signaling pathway. We therefore measured the surface area covered by the vasculature, the proportion of vascular-associated newborn cells, and analyzed VEGF and Flk-1 protein expression in the hippocampus of a control, chronically stressed and recovery group of rats. Our results show that 32% of the proliferating cells in the rat hippocampus is vascular associated. This percentage was significantly decreased by chronic stress. Interestingly, after 3 weeks of recovery, the decreased proliferation not associated with the vasculature, was more effectively restored than vascular-associated proportion of proliferating cells. VEGF protein was expressed in high densities in GFAP-positive astrocytes located in the hilus, with VEGF-positive end feet extending into and often contacting the granule cells. After chronic stress, both VEGF and Flk-1 protein levels were significantly decreased in the GCL, and again recovered after 3 weeks. This demonstrates that changes in angiogenic factors are implicated in the decreased adult proliferation found after chronic stress.
Chapter 5

Introduction

Exposure to stress decreases cell proliferation in the adult hippocampal dentate gyrus (DG) (Gould et al., 1997; Gould et al., 1998; Fuchs et al., 2001; Tanapat et al., 2001; Czeh et al., 2002; Heine et al., 2004). Circulating glucocorticoids (GCs) are believed to be instrumental in the structural and functional hippocampal changes after chronic stress (Sapolsky et al., 1985; Magarinos et al., 1996; Joels, 2001; Lucassen et al., 2001; McEwen, 2001). However, the exact mechanisms responsible for the suppressed cell proliferation remain largely unknown.

Recent evidence has shown that adult proliferation takes place near the local microvasculature of the hippocampus (Palmer et al., 2000; Louissaint et al., 2002; Fabel et al., 2003; Yamashima et al., 2004), while also both angiogenesis and neurogenesis can be modulated by similar stimuli (Jin et al., 2000; Fabel et al., 2003). This suggests that regulatory signals for adult proliferation may at least partially be derived from the endothelium.

The vascular endothelial growth factor (VEGF) is a major angiogenic factor (Jakeman et al., 1993; Flamme et al., 1995; Rosenstein et al., 1998) that can also exert direct neurotrophic and neuroprotective effects (Hayashi et al., 1998; Issa et al., 1999; Silverman et al., 1999; Jin et al., 2000; Sondell et al., 2000; Matsuzaki et al., 2001; Oosthuyse et al., 2001; Svensson et al., 2002; Widenfalk et al., 2003). Moreover, changes in VEGF concentration and VEGF receptor expression were shown to result in altered cell proliferation and survival rates both in vitro (Sondell et al., 1999; Jin et al., 2002) and in vivo (Sondell et al., 1999; Jin et al., 2002; Fabel et al., 2003). VEGF can interact with 2 receptors the fms-like tyrosine kinase (VEGF-R1, Flt-1) and the fetal liver kinase receptor (VEGF-R2, Flk-1) (Neufeld et al., 1999). In the adult brain, Flk-1 rather than Flt-1 is abundantly present (Yang et al., 2003).

Corticosteroids are well known inhibitors of angiogenesis and have also been extensively studied for e.g. their use in anti-tumor treatment (Folkman, 1972; Auerbach & Auerbach, 1994; Folkman, 1995). Furthermore, studies involving different cell types have revealed that steroids like estrogen, dexamethasone and corticosterone, are all able to regulate VEGF and / or VEGF receptor mRNA expression (Cullinan-Bove & Koos, 1993; Klekamp et al., 1997; Nauck et al., 1998; D'Angio et al., 1999; Machein et al., 1999; Mueller et al., 2000; Halaby et al., 2002; Sibug et al., 2002; Mallet et al., 2003; Clerch et al., 2004). These effects seem GR mediated, since e.g. inhibitory actions of dexamethasone could be reversed by GR antagonist application (Heiss et al., 1996; Gloddek et al., 1999).
In this study, we therefore questioned whether the effects of chronic stress on new cell proliferation are (at least in part) mediated through effects on the vasculature, and whether chronic stress affects an angiogenic-signaling pathway. Therefore, we measured the surface area covered by the vasculature, the number of vascular-associated proliferating cells, and analyzed VEGF and Flk-1 receptor expression immunocytochemically in the hippocampus of control and chronically stressed rats, and in rats that were allowed to recover 3 more weeks after stress. To determine the proportion of newborn cells proliferating near the vasculature, sections were double-labeled immunocytochemically with the vascular marker Reca (rat endothelial cell antigen) and the proliferation marker Ki-67.

Material & Methods

Animals

All animals were male Wistar rats (Harlan, the Netherlands) studied at the age of 10 weeks. Two rats were housed per cage under controlled conditions (21°C room temperature, 60% humidity, lights on from 8.00-20.00h) with food and water available ad libitum. Rats were randomly assigned to the control (n = 10), chronic stress (n = 11) or stress + recovery (n = 10) group. The local animal ethical committee of the University of Amsterdam approved of all experiments.

Stress protocol

Animals were chronically stressed according to a multiple unpredictable stress paradigm as described earlier in detail (Herman et al., 1995; Heine et al., 2004). Briefly, rats were exposed to different stressors twice daily for 21 days, consisting of (cold) immobilization, forced (cold) swimming, crowding, isolation and vibration. The animals that were allowed to recover were handled for another three weeks after stress exposure. Control rats were also handled twice daily, to exclude effects of handling of the stressed rats. On forehand, 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.

Brain tissue preparation

Animals were deeply anaesthetized in the morning by i.p. injection of pentobarbital sodium salt (Nembutal 1 ml/kg bodyweight; 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 40 μm 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 use.

**Reca / Ki-67 double immunocytochemistry**

**Day 1:**

Free-floating sections were washed in 0.1M Tris-Buffered Saline (TBS) pH 7.6. To block endogenous peroxidase activity, 1.5% hydrogen peroxide in TBS was added for 15 min. After washing, sections were blocked with 2% milk (Elk, Campina Melkunie, Eindhoven, Netherlands) in TBS for 1 hr, to reduce nonspecific binding, followed by overnight incubation (4°C) of primary antibody mouse α-Reca-1 (Sero-tec, Breda, The Netherlands) diluted in 0.25% gelatine / 0.1% Triton X-100 in TBS (Supermix).

**Day 2:**

After washing, 0.5% normal goat serum / 0.3% Triton X-100 in TBS (TBS+) was added to block nonspecific binding. Incubation of secondary antibody biotinylated IgG α-mouse (Amersham Life Sciences, Den Bosch, Netherlands) in TBS+ was performed for 2 hrs at RT. Then sections were washed, blocked for 10 min in TBS+ and the avidin biotin peroxidase complex solution (ABC; 1:1000, Vectastain Elite, Brunschwig Chemie, Amsterdam, The Netherlands) was added for 2 hrs. Color development was performed with diaminobenzidine (0.50 mg DAB / ml Tris/HCL, 0.01% H₂O₂) for 25-30 min. After several rinses, the sections were mounted onto Plus glass slides (Menzel) and dried overnight at 37°C.

**Day 3:**

The slides were rinsed with TBS. Ki-67 immunolabeling was done as described before (Heine et al., 2004). Briefly, tissue was pretreated in a domestic MW oven (Samsung M 6235) for 15 min (5 min at 800 W, 400 W and 260 W). Endogenous peroxidase activity was blocked by 15 min 1.5% hydrogen peroxide treatment in TBS. To reduce nonspecific binding, 2% milk in TBS was applied for 30 min. Sections were then incubated overnight with the primary antibody polyclonal rabbit α-Ki-67 (Novocastra, New Castle, UK, 1:2000) diluted in Supermix.

**Day 4:**

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 1% BSA / TBS 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 hrs incubation with ABC (1:1000). Color development was performed with diaminobenzidine (0.3 mg DAB / ml Tris/HCL) 0.001% \( \text{H}_2\text{O}_2 \) and 0.04% Nickel for 6-10 min, after which sections were washed, dehydrated, passed through xylene and coverslipped with Entellan.

**VEGF and Flk-1 immunocytochemistry**

Free-floating sections were washed in TBS pH 7.4. Endogenous peroxidase activity was blocked by adding 1.5% \( \text{H}_2\text{O}_2 \) in TBS for 15min. After washing, nonspecific binding was blocked with 5% normal goat serum / 0.3% Triton X-100 in TBS (TBS++) for 1 hr at RT. Then, sections were incubated overnight with the primary antibody polyclonal rabbit \( \alpha\)-Flk-1 (1:500) or \( \alpha\)-VEGF (1:3000, both from Santa Cruz Biotechnologies, Heerhugowaard, The Netherlands) in TBS++. The next day, sections were washed, blocked with TBS++ for 15 min, followed by secondary antibody incubation with biotinylated IgG \( \alpha\)-rabbit (1:250) in TBS++ for 1.5 hrs. Then, labeling was continued and finished as described for Reca / Ki-67 double labeling (day 4) starting from ABC treatment onwards. Omission of the first antibody gave no signal (not shown).

**GFAP and VEGF immunofluorescence.**

After washing free floating sections in 0.1M TBS pH 7.6, non-specific binding was blocked with TBS++ for 1hr at RT. Then, sections were incubated overnight with the primary antibody mouse monoclonal \( \alpha\)-GFAP (1:500, Chemicon International, Amsterdam, the Netherlands) and rabbit polyclonal \( \alpha\)-VEGF (1:200) in TBS++. The next day, sections were washed, blocked with TBS++ for 15 min, the first antibodies were subsequently detected with Alexa Fluor 546 and Alexa Fluor 488, respectively, and embedded in Vectashield (Vector Laboratories).

**Quantification and stereology**

To obtain an optimal view of the three-dimensional location of the proliferating cells, confocal analysis would have been useful. To that end, pilot studies were first performed in which we compared confocal (Fig. 1E) with conventional light microscopical analysis. These yielded highly comparable results (data not shown). Since the latter technique is more convenient and less time consuming, we selected this approach for the
rest of the study.

Serial sections (40 µm, every 12th section) of one hemisphere were taken for stereological quantification of Ki-67-positive cell numbers. The numbers of newborn cells were assessed in the main hippocampal sub-regions, the hilus and SGZ, in a stereological approach over the entire rostro-caudal extent of the hippocampus. Ki-67 positive cells or clusters of these cells were considered ‘vascular-associated’ (VA) when they were at no more than one nucleus distance from a blood vessel, and were scored separately. The proportion of proliferation associated with the vasculature for one treatment group was estimated from the mean calculated percentages (of VA Ki-67 positive cells) for each animal in that group.

To calculate the proportion of surface area covered by the microvasculature, Reca immunolabeled serial sections (every 12th) were photographed with a Nikon Coolpix 4500 digital camera connected to a Zeiss Axiophot microscope (10x objective). In view of possible anatomical differences along the septotemporal axis (Grivas et al., 2003), this was quantified in a stereological approach. Pictures were then transferred to a Macintosh computer, on which a High Pass filter (radius 10) was applied using the program Adobe Photoshop 5.5 to obtain continuous-tone gray scale images. Then, Tiff files were transferred to 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). To make high contrast black-and-white images, a threshold routine with a fixed value was applied, after which binary images were obtained. Noise was reduced using ‘dilation’ and ‘erosion’ routines (Set Iterations: 1). The SGZ – Hilus area was outlined and the surface area measured, as well as the numbers of black and white pixels within that region. The proportion of black pixels was used as a measure for the surface area covered by the blood vessels in the total SGZ-Hilus region (See Fig. 1D). To further address whether the presence of Ki-67-positive cells could possibly have contributed, or interfered with the accuracy of the image analysis of the blood vessel density, pilot studies were performed in which we compared single labeled and doubled sections, with negligible differences found (data not shown).

Analysis of VEGF-positive astroglia in the hilar region was done in the DAB-immunolabeled sections, using a Macintosh computer program for image processing, called “μIMAGE” (developed by P.C. Diegenbach, University of Amsterdam, The Netherlands), as described earlier by Van Raamsdonk et al. (1996). Individual sections were viewed on a video monitor (604 x 576 pixel array) via a black / white CCD video camera (High Technology Holland, Eindhoven, The Netherlands) connected to a Zeiss
Pan II: Stress - Vasculature

Axiophot microscope (100× NA 1.3 objective). The scanned area in the astroglial cell somata was at most 40 μm². More than 100 astroglia (approximately 10 per section) were measured over the serial sections (every 12th) to calculate the mean extinction per animal. The collected data represent mean integrated absorbance values (MIA). Images were corrected for background absorbance by subtraction of a blank image that was taken near the specimen on the slide.

Consistent with the expected distribution of a membrane receptor, Flk-1 immunolabeling showed a punctuate appearance in the GCL, hilus and the molecular layer. Signal was quantified by assessing the number of expression spots within selected counting frames. The punctuate appearance of VEGF immunolabeling in the GCL was analysed the same way, in addition to the number of VEGF-positive astroglial cells in the hilus. Counting frames (hilus / mol. l.: 125 × 125 μm²; GCL: 25 × 25 μm²) were placed according to the schematic representation in Fig. 1D. The images were visualized using an Olympus microscope (40× NA 0.70 objective). VEGF-positive astroglial cells, and the VEGF and Flk-1 expression spots were counted 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). The hippocampal intersections Bregma 3.30 and 4.80 mm were used to calculate the mean density of profiles in the different areas within one animal.

Statistics

Statistical analysis was performed using one-way analysis of the variance (ANOVA) and an unpaired Student’s t-test with a two-tailed p-value. Differences were 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

Three weeks of unpredictable stress significantly decreased the numbers of proliferating cells in the hippocampal DG, as we reported earlier (Heine et al., 2004). We here investigated if chronic stress also influenced the proportion of newborn cells associated with the vasculature. Blood vessels were visualized with a brown and the newborn cells with a black precipitate (Fig. 1A, see Appendix). A large part of the Ki-67 positive cells appeared in clusters, mainly in the SGZ. Total numbers of vascular-associated (VA) and
non-associated (NA) Ki-67 positive cells are presented in Fig. 3A and B, respectively. In both the hilus and the SGZ, numbers of the VA newborn cells (Fig. 3A; ANOVA hilus: $p = 0.003, F = 7.31$; ANOVA SGZ: $p = 0.0006, F = 10.22$; ANOVA hilus + SGZ: $p = 0.0003, F = 11.33$; t-test hilus: $p = 0.0005, F = 1.89$; t-test SGZ: $p = 0.0006, F = 1.22$; t-test hilus + SGZ: $p = 0.0003, F = 1.21$) and NA newborn cells (Fig. 3B; ANOVA hilus: $p = 0.015, F = 5.07$; ANOVA SGZ: $p = 0.0003, F = 11.84$; ANOVA hilus + SGZ: $p < 0.0001, F = 14.88$; t-test hilus: $p = 0.003, F = 1.27$; t-test SGZ: $p < 0.0001, F = 1.19$; t-test hilus + SGZ: $p < 0.0001, F = 1.31$) were significantly decreased after chronic stress. When animals were allowed to recover for 3 more weeks after chronic stress, VA and NA proliferation rates returned towards control levels in both the hilus and SGZ (Fig. 3A and B). However, when taken together, in the hilus and SGZ, VA proliferation was still significantly reduced compared to control rats (t-test, $p = 0.04, F = 3.18$), whereas NA proliferation of the recovery group was normalized and significantly different from the chronic stress group (t-test, $p < 0.02, F = 1.15$). Chronic stress affected the VA cells to a significantly larger extent than NA cells; mean total numbers of VA newborn cells decreased by 54% in the hilus, compared to a 35% decrease of the NA newborn cells. Comparable differences between these populations of new cells were seen in the SGZ, as 38% reductions in VA, and 24% reductions in NA proliferation were measured after chronic stress.

Figure 3A:

![Graph showing Ki-67 proliferation in control, chronic stress, and recovery groups in the hilus, SGZ, and hilus + SGZ.](image)

VA proliferation was still significantly less than in control rats (A: $p = 0.04$), whereas NA proliferation was already significantly increased compared to the chronic stress group (B: $p < 0.02$). Proliferating cell numbers are expressed as the estimated mean total number ($\pm$ 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).
Additionally, the proportion of VA cells was expressed as percentage of all the Ki-67 positive cells, as shown in Figure 4. After chronic stress, VA cells made up a significantly smaller proportion of the total number of proliferating cells (Fig. 4). VA proliferation was decreased in the hilus (ANOVA $p = 0.25$, $F = 1.45$) and significantly reduced in the SGZ ($t$-test $p < 0.04$, $F = 2.81$) and hilus + SGZ ($t$-test $p = 0.02$, $F = 1.24$; ANOVA $p = 0.05$, $F = 3.35$). After an additional three weeks of survival, no significant differences with the controls were seen anymore. Altogether, our data show that after chronic stress, VA proliferation of new cells is stronger reduced than the NA proliferation.

Figure 4: Chronic stress reduced the percentage of VA proliferation in the SGZ ($p < 0.04$) and hilus + SGZ ($p = 0.02$). After three additional weeks of survival, the proportion of VA proliferating cells was not different from controls anymore. The proportion of VA cells is expressed as the mean percentage (± SEM) of Ki-67 positive cells close to blood vessels compared to the total number of proliferating cells in that same hippocampal region; calculated for the control ($n = 10$), chronically stressed ($n = 11$) and recovered 10-weeks-old rats ($n = 6$).

To address whether chronic stress had affected the vascular bed itself and thereby possibly indirectly influenced the chance of association with the adult generated cells, and hence VA proliferation, the surface area covered by the microvasculature was measured in Reca immunolabeled sections (Fig. 1D, see Appendix). The proportion of surface area covered by the vasculature was about 11%, which was not changed after chronic stress.
(Control (n = 7): 10.9 ± 0.6%; mean of total measured area: 7.54 ± 0.31 mm² <-> CS (n = 11): 11.3 ± 0.4%; 7.72 ± 0.52 mm²).

We further tested whether chronic stress affected the expression of the growth factor VEGF and its receptor Flk-1. VEGF protein expression was present in high densities in both cytoplasm and processes of the GFAP-positive astrocytes in the hilar region, and to a lesser extent in the GCL (Fig. 1B + 2, see Appendix). VEGF-positive glial end feet extended into the GCL, where a punctuate expression pattern was observed on the somata of the granule cells (Fig. 1B + 2, arrows in C and D, see Appendix). Astroglia cell number in the hilar regions and the mean absorbance values in their somata were not changed after chronic stress (Table 1). However, the VEGF expression levels in the astrocytes were significantly increased after 3 weeks of recovery. In the dentate granule cells, the punctuate VEGF expression pattern measured was significantly reduced after chronic stress, but recovered after 3 weeks of rest (Table 1).

Table 1:

<table>
<thead>
<tr>
<th>VEGF density</th>
<th>astrocyes in hilus</th>
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<tbody>
<tr>
<td>per 100,000/μm²</td>
<td>number</td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>0.0008</td>
</tr>
<tr>
<td>Chronic Stress (n=10)</td>
<td>0.03</td>
</tr>
<tr>
<td>Recovery (n=6)</td>
<td>144 ± 4</td>
</tr>
<tr>
<td>t-test p-value</td>
<td>96 ± 8 (n=9)</td>
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<tr>
<td>Control</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Chronic Stress</td>
<td>120 ± 12</td>
</tr>
<tr>
<td>t-test p-value</td>
<td>Control</td>
</tr>
<tr>
<td>Chr Stress</td>
<td>0.13</td>
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Table 1: VEGF protein expression in the GCL is expressed as the mean number of expression dots per 100,000/μm². Individual VEGF-positive astrocytes in the hilar region are analyzed for their expression levels, and expressed as mean extinction values. Further, the number of VEGF-positive astrocytes are counted, and expressed as the mean number per 100,000/μm² hilar area.

Immunolabeling for Flk-1 revealed a punctuate expression pattern in the granule cell layer, molecular layer and the hilar region (Fig. 1C). In all these areas, expression was significantly reduced after chronic stress. After 3 weeks of rest, the expression level in the hilar region was still significantly decreased (Table 2), whereas normal levels were present in the other subregions.

Table 2: Flk-1 protein expression is expressed as the mean number of expression dots per 100,000/μm² for the different hippocampal regions.
Table 2:

<table>
<thead>
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<th>Fli-1 (VEGF-receptor 2) density</th>
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<tr>
<td></td>
<td></td>
<td>DG</td>
<td>hilus</td>
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<td>Control (n=10)</td>
<td>ANOVA p-value</td>
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<td>0.0002</td>
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<td></td>
<td>F-value</td>
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<td>Chronic Stress (n=7)</td>
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<td></td>
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<td>97 ± 4</td>
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<td></td>
<td>80 ± 7</td>
<td>18.4 ± 1.1</td>
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<td>Recovery (n=4)</td>
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<td></td>
<td></td>
<td>Control</td>
<td>Chr Stress</td>
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<td>88 ± 3</td>
<td>25.4 ± 0.8</td>
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<td>0.22</td>
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Discussion

We report here that about one third of all proliferating (Ki-67 positive) cells in the adult rat DG are in close anatomical association with the microvasculature. Interestingly, chronic stress affected this population of newborn cells to a significantly larger extent than the non-associated cells. Importantly, no stress-induced changes were found in the surface area covered by blood vessels. Quantitative immunocytochemistry further revealed decreased VEGF protein expression, as well as decreased Flk-1 expression in the GCL. The latter was also decreased in the hilus and molecular layer of the DG. Together, this not only favors the concept that blood vessels are part of a unique microenvironment to support hippocampal neurogenesis, but also indicates that this microenvironment is sensitive to stress. These changes in angiogenic growth factor expression may, at least partly, be responsible for the decrease in adult proliferation after stress.

Previous studies have firmly established a decreased proliferation in the DG of various species following short-lasting stress or GC exposure (Gould et al., 1991; Galea et al., 1997; Gould et al., 1997; Gould et al., 1998; Gould & Tanapat, 1999; Lemaire et al., 2000; Tanapat et al., 2001). Since we presently studied only the proliferating cells, the phenotype of this heterogeneous population of cells is unknown. However, we have shown before (Heine et al., 2004), that after 3 weeks of survival, the large majority, i.e., at least 50% of the newborn cells become neurons. Others reported similar data as well (Kempermann et al., 2003). The present stress-induced reductions in proliferation are generally transient as they recover e.g. acute stress within 24 hours (Heine et al., 2004). It is only after prolonged stress, that lasting effects on structural parameters and adult cell proliferation become apparent (Pham et al., 2003; Heine et al., 2004). These changes are not reversed easily and only partially recovered after 3 more weeks of survival (Heine et
Since acutely stressed animals showed recovered Ki-67 cell numbers after 24 hrs already, the present study focused on chronic stress alone.

The mechanism through which chronic stress, or elevated cort levels, affects new cell birth is still poorly understood. So far, no glucocorticoid receptors have e.g. been identified on the newborn precursor cells in the DG (Cameron et al., 1993a). Since the granular cell layer contains high concentrations of GR and MR, corticosteroid signaling has been proposed to occur indirectly via e.g. neighboring mature granule cells, through astrocytes or radial glial cells (Doetsch, 2003). Alternatively, various growth factors, and even upstream mechanisms involving glutamate and NMDA receptor mediated actions, have been implicated as well (Cameron et al., 1995; Reagan & McEwen, 1997). However, no NMDA receptors have been identified on precursor cells either (Cameron et al., 1993a).

The highly selective and rare occurrence of neurogenesis in the adult hippocampal DG and subventricular zone, has led to the recent suggestion in literature, that new cell birth only takes place in a unique microenvironment, that supports adult proliferation and neuronal differentiation (Doetsch et al., 1999; Palmer et al., 2000; Louissaint et al., 2002; Palmer, 2002; Seki, 2003). Palmer et al. (2000) proposed that hippocampal neurogenesis occurs in an angiogenic niche, in which angiogenesis and neurogenesis happen concurrently. This concept was extended by Louissaint et al., (2002) showing that the angiogenic bed in the adult songbird neostriatum represents a spatial target for migrating neuroblasts, as well as a source for trophic support upon their arrival. Yamashina et al. (2004) even demonstrated that the vascular adventitia could serve as a source of neuronal progenitor cells in primates. Consequently, we hypothesized that the negative effects of chronic stress on new cell proliferation involve this angiogenic niche, and that they are, at least in part, mediated through changes in the vasculature bed or an angiogenic-signaling pathway.

Here we show that 32% of the proliferation in the rat dentate gyrus is vascular-associated, which is in the same order of magnitude as the 37% found previously by Palmer et al., (2000). The small difference is probably due to the somewhat stricter criteria we set for vascular association and our use of the proliferation marker Ki-67 (Gerdes et al., 1984; Endl & Gerdes, 2000) as opposed to BrdU (Palmer et al., 2000). Since α-BrdU identifies dividing cells in S phase as well as their progeny, and α-Ki-67 all actively cycling cells, these methods visualize two different populations. Either way, our results not only confirm earlier data showing that a large proportion of the adult generated cells proliferate in close association with the microvasculature (Palmer et al., 2000; Louissaint et al., 2002), but also extend these findings by showing that chronic stress reduces the
proportion of VA proliferating cells. We furthermore show that proliferation not associated with the vasculature is more effectively restored by a 3-weeks recovery period than VA proliferation. The reduced numbers in the SGZ seem mainly responsible for this lack in recovery. Between hilus and SGZ, different ratios between glial, oligodendrocytic and neuronal precursor populations seem to exist (Steiner et al., 2004). One could speculate, since a higher proportion of glia cells proliferate in the hilus compared to the SGZ (Cameron et al., 1993b), while in the SGZ, a higher proportion of newborn cells proliferate near the vasculature, that vascular-associated proliferating cells in the SGZ are more likely to become granule cells, and therefore more vulnerable for chronic stress-induced changes. Clearly, this awaits further research.

As a possible explanation for these stress effects on VA proliferation, VEGF is an important candidate as it is a common regulating factor for both neurogenesis and angiogenesis. Initially, VEGF was described for its role in angiogenesis by providing the signal for inducing and guiding growth of new vessels (Breier et al., 1992; Jakeman et al., 1993; Yancopoulos et al., 2000) and increasing blood vessel permeability (Flamme et al., 1995; Augustin, 1998; Dvorak, 2000). Consistent with this, VEGFR1/Flt-1 and VEGFR2/Fk-1 expression were primarily ascribed to endothelial cells (Neufeld et al., 1999). Currently, VEGF is also known for its neurotrophic and neuroprotective actions (Silverman et al., 1999; Sondell et al., 1999; Jin et al., 2000; Sondell et al., 2000; Matsuzaki et al., 2001; Carmeliet & Storkebaum, 2002). Likewise, expression of VEGF and/or its receptor Flk-1 has been reported in hematopoietic stem cells (Gerber et al., 2002), retinal progenitor cells (Yang & Cepko), hippocampal neuronal progenitor cells (Palmer et al., 2000; Jin et al., 2002), and in all neural stem cell lines that are derived from hippocampus, subventricular zone and olfactory bulb (Maurer et al., 2003). Furthermore, Louissaint (2002) showed in adult songbirds that VEGFR2/Fk-1 stimulation is necessary for the recruitment of neuronal progenitors to the higher vocal center. Jin et al. (2002) even demonstrated that VEGF can stimulate cell proliferation in the SGZ and SVZ, increasing the generation of new neurons, astroglia and endothelial cells. Moreover, Fabel et al. (2003) recently showed that VEGF is necessary for the exercise-induced increase in hippocampal neurogenesis. All these studies confirm the close association between VEGF and hippocampal neurogenesis.

We observed VEGF protein expression predominantly in the GFAP-positive astrocytes, and in lower amounts in neuronal cells, as is clear from the punctate pattern over the granule cells in Fig 2. This is in line with Ogunshola et al. (2000), who described VEGF in relation to early postnatal cortical development. Initially, VEGF is found in a
neuronal cell population, but later, as the vascular bed begins to stabilize, this switches to mainly glial expression, during which neuronal VEGF expression is reduced to very low basal levels (Ogunshola et al., 2000). Under hypoxic conditions, it is known that both glial as well as neuronal expression are strongly upregulated throughout the adult brain (Shweiki et al., 1992; Xu & Severinghaus, 1998; Issa et al., 1999; Kuo et al., 1999; Ogunshola et al., 2000), including the hippocampal DG (Marti & Risau, 1998; Marti et al., 2000). Under naïve intact conditions, VEGF-expressing glial end feet not only invade blood vessel walls, thereby probably maintaining the vasculature (Alon et al., 1995; Ilan et al., 1998), but also enter the granular cell layer, as we found as well; this likely reflects trophic support of the main DG cell layer.

In the present study, Flk-1 expression showed a punctuate distribution over the granule cell layer. In our hands, no expression was observed in any of our animals for the other receptor, Flt-1 (data not shown), which is consistent with earlier reports showing that mature, naïve neurons do not express Flt-1 (Yang et al., 2003), but can be induced to do so following ischaemia / hypoxia (Lennmyr et al., 1998). In the adult intact brain however, Flk-1 rather than Flt-1, expression is found in abundant quantities (Yang et al., 2003), and is the predominant receptor in both neonatal and adult brain.

Corticosteroids are among the most intensively studied inhibitors of angiogenesis (reviewed by (Auerbach & Auerbach, 1994)), and have been widely used as a strategy to target tumor blood supply e.g. (Folkman, 1972, 1995). Although the exact mechanism is poorly understood, there is evidence that corticosteroids can inhibit VEGF activation in some tissues. For example, corticosteroids inhibit VEGF expression in human vascular smooth muscle cells (Nauck et al., 1998), in osteoblasts (Harada et al., 1994) and in mouse adrenals (Mallet et al., 2003). In the latter study, Flk-1 mRNA was also decreased, whereas Flt-1 remained constant (Mallet et al., 2003). The dexamethason induced suppression of VEGF expression in glioma (Heiss et al., 1996; Machein et al., 1999) and pituitary folliculostellate cells e.g. (Gloddek et al., 1999) could be reversed by GR antagonists, indicating involvement of the GR as well.

We questioned whether 3 weeks of chronic stress exposure, which was shown before to elevate basal corticosterone levels (Heine et al., 2004), would change hippocampal VEGF and / or Flk-1 protein expression, and found decreases in both protein levels in the GCL what returned to normal levels after 3 weeks of recovery. The decreased VEGF protein levels in the GCL likely follows the chronic stress induced decrease in Flk-1 receptor expression, as a decrease in receptors would leave less binding sites available. VEGF expression in the soma of the astrocytes residing in the hilar region was not changed.
after chronic stress, but was surprisingly increased after 3 weeks of recovery. This increase in overall astrocytic VEGF expression (mean extinction), while no changes in the number of astrocytes is present (Table 1), suggests a supportive role for astrocytes in the recovery of proliferation in the stress impaired dentate gyrus (Heine et al., 2004). This awaits however, further research.

So far, it is unresolved whether the chronic stress-induced VEGF changes are responsible for the decreased (VA) hippocampal proliferation. Our data on the surface area covered by blood vessels, exclude the possibility that VEGF, through Flk-1, changed overall blood vessel structure and thereby the VA proliferation. However, other pathways can be considered. Firstly, VEGF binding to Flk-1 is known to activate specific intracellular signaling cascades, which involve a.o. phosphatidylinositol 3-kinase (PI3K) and MEK extracellular signal-regulated kinase (ERK) (Thakker et al., 1999; Matsuzaki et al., 2001; Cross et al., 2003). In turn, these regulate expression of important regulators in the G_1 phase of the cell cycle (Lavoie et al., 1996; Weber et al., 1997; Gille & Downward, 1999; Jones & Kazlauskas, 2001; Zhu et al., 2003). By using BrdU, Zhu et al (2003) indeed showed that the neuroproliferative effect of VEGF was associated with an upregulation of cyclin D_1, cyclin E and E2F transcription factors in vitro, all of which are necessary for the progression through the G_1 phase and the G_1/S transition (Yoshikawa, 2000; Jones & Kazlauskas, 2001). In addition, VEGF-stimulated proliferation of endothelial cells appeared to involve the same pathways (Thakker et al., 1999; Wu et al., 2000; Suzuma et al., 2002). Consequently, a reduced new cell turnover rate after stress might be due to a suppressed PI3K/Akt and / or MEK/ERK signalling in proliferating cells, which in turn, might be caused by a decline in neurotrophic factors, such as VEGF and / or Flk-1.

A second possibility is that decreased VEGF levels might have directly affected the microenvironment to support generation of new neurons, by e.g. perturbing blood vessel permeability (Palmer et al., 2000; Louissaint et al., 2002). This could affect brain access for other trophic factors, like FGF-2 (Wagner et al., 1999) and IGF-1 (Aberg et al., 2003). It is interesting in this respect, that running, a robust stimulator of neurogenesis, was recently shown to increase many circulating growth factors in parallel (Asano et al., 1998; Schobersberger et al., 2000; Trejo et al., 2001; Campuzano et al., 2002), whereas the enhance in neurogenesis could be inhibited already by blocking VEGF alone (Fabel et al., 2003).

Thirdly, VEGF could have influenced local vasculature properties through reductions in brain-derived neurotrophic factor (BDNF) expression, which factor was upregulated in parallel with the VEGF-induced neurogenesis in songbirds (Louissaint et
and abundantly expressed at the vascular adventitia of the SGZ following ischemia in primates (Yamashima et al., 2004). BDNF is required for basal neurogenesis (Lee et al., 2002) and can be modulated by astrocytes and endothelial cells to support neuronal differentiation (Kirschenbaum & Goldman, 1995; Leventhal et al., 1999; Song et al., 2002). As chronic stress (Smith et al., 1995) or GC exposure (Schaaf et al., 1997; Schaaf et al., 1998) were already shown to decrease BDNF levels, future studies might reveal whether BDNF expression is also affected by the present chronic stress paradigm or is related to the current VEGF changes.

In conclusion, we show that 32% of the adult hippocampal cell proliferation is vascular associated and that chronic stress affects this VA proliferation more than the NA proliferation. This cannot be ascribed to a smaller vascular bed, but does suggest the involvement of an angiogenic related change after chronic stress. Indeed, protein levels of VEGF and its receptor Flk-1, which are known for their role in both angiogenesis and neuronal survival, were decreased after chronic stress. However, it remains open whether the increased corticosteroid levels are directly responsible for their down-regulation. This study supports the concept that the vasculature is part of a unique microenvironment to support neurogenesis, and shows that it can be affected by chronic stress.

Acknowledgements
The authors would like to thank E.A. Koop (University Medical Center, Utrecht) for providing antibodies and W. van Raamsdonk (University of Amsterdam, Amsterdam) for valuable discussion.