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

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Chapter 6

Age-Related Changes in the Vascular-Associated Population of Actively Cycling Cells in the Rat Dentate Gyrus

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Abstract

Hippocampal neurogenesis decreases profoundly, and already early, during aging. The factors responsible for this are, however, unknown. Following the observation that a large part of the adult-generated cells is closely associated to the vasculature, recent studies have focused on the involvement of the vasculature and vascular-related factors. Since aging causes a.o. haemodynamic and vascular changes, we address here vascular involvement in the age-related decline in proliferation and studied proliferating cells in relation to their location near the vasculature. In the aging dentate gyrus (DG), we found a decrease in the proportion of vascular-associated proliferation, in a regionally dependent fashion, whereas, the surface area of the vascular bed increased in old age. Furthermore, the proliferative activity of both the vascular- and nonvascular-associated newborn cells was analyzed by double labeling with BrdU and Ki-67. Changes in the progenitor population with age were further validated using immunocytochemistry for p27Kip1, a cyclin dependent kinase inhibitor involved in G1 arrest. Between 2 and 6 weeks of age, the proportion of double labeled cells increased significantly, which was paralleled by an increased occurrence of proliferative clusters, indicating an increased number of actively cycling cells at these ages. In contrast, in middle-aged and old rats mainly “silent”, single BrdU labeled newborn cells were present, while also the number of p27Kip1-positive cells in the DG decreased indicating a decline in the available progenitor cell population as well. Our results support the importance of the vasculature in the generation of new granule cells. They indicate that the age-related decline in proliferation is not simply due to changes in the microenvironment of the vasculature, but also the result of changes in intrinsic properties in the progenitor cell population.
Introduction

Aging strongly reduces the number of proliferating cells in the hippocampal dentate gyrus (DG) (Seki & Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998). Notably, this occurs already from relatively young ages onwards (Heine et al., 2004a). Although, various studies have shown parallels between changes in neurogenesis and changes in cognitive performance, that suggest a role in learning, the functional role of the adult generated cells is currently unclear (Gould et al., 1999; van Praag et al., 1999; Kempermann, 2002; Kempermann & Gage, 2002). Consistent with this idea, the age-related decrease in neurogenesis has been linked to decreased cognitive performances in old rats (Bizon & Gallagher, 2003). A better understanding of the factors involved in the age-related decline in proliferation might help to explain age-related cognitive decline. Presently it is unclear whether aging affects the number of progenitors present, their intrinsic proliferative potential, or the microenvironment that allows these cells to proliferate and differentiate within distinct local niches.

Recent evidence has shown that adult proliferation takes place in close association with the local microvasculature of the hippocampal dentate gyrus and the subventricular zone (SVZ), the two main areas where adult neurogenesis is found (Palmer et al., 2000; Louissaint et al., 2002; Fabel et al., 2003). In addition, angiogenesis and neurogenesis can be modulated by similar stimuli (Jin et al., 2002; Fabel et al., 2003). Fabel et al (2003) e.g. showed that peripheral blockade of the vascular endothelial growth factor (VEGF) abolished the running-induced increase in hippocampal proliferation (Fabel et al., 2003). In a previous study, we showed that the chronic stress-induced decrease in vascular-associated proliferation is paralleled by a reduced expression of both the angiogenic factor VEGF as well as its receptor Flk-1 (Heine et al., 2004c). This indicates that regulatory signals for adult proliferation involve, at least partially, also the vasculature.

Aging is known to cause prominent haemodynamic and structural vascular changes, including thickening of the basal lamina (Kalaria, 1996), alterations in transport over the blood brain barrier (Shah & Mooradian, 1997) and decreased cerebral blood flow (Noda et al., 2002). Whether age-related changes in the vasculature are involved in the decline in proliferation during aging is, however, still unknown.

We therefore studied the proliferative activity of adult-generated cells in relation to their association with the vasculature, and addressed the possible involvement of the vasculature in the age-related decline in proliferation, by studying changes in the vascular bed during aging and whether the vascular-associated proliferation changes in parallel.
To that end, the surface area covered the blood vessels was measured, together with the proportion of vascular-associated proliferating cells in young (2w), young-adult (6w), middle-aged (12m), and old (24m) Wistar rats. Additional confocal microscopy analysis of two proliferation markers (Ki-67 and BrdU) was used to analyze temporal dynamics of the clusters of proliferating cells in relation to their association with the vasculature. Finally, to address whether aging directly affected the population of progenitor cells in the DG, p27Kip1 was immunocytochemically studied. P27Kip1 is a cyclin dependent kinase inhibitor (Sherr, 1994), which can inhibit cell cycle progression. Furthermore, p27Kip1 expression is low in post-mitotic neurons and considered a key regulator in proliferative cell populations (van Lookeren Campagne & Gill, 1998; Doetsch et al., 2002; Heine et al., 2004b). Since we previously found a clear and restricted location of this progenitor marker in the neurogenic SGZ of the hippocampus as well as changes after chronic stress (Heine et al., 2004b), we included it in relation to aging of the SGZ.

Material & Methods

Animals

Wistar rats (male) were studied at 2 weeks (young) (n = 10), 6 weeks (young adults) (n = 9), 12 months (middle aged) (n = 10), and 24 months (old) (n = 10) of age. Middle-aged and old rats were obtained from the aging colony of the Institute of Physiological Psychology, University of Düsseldorf, Germany. After transport to our facilities, the animals were first left undisturbed for 2 weeks before experiments started. Animals were single housed under controlled conditions (21°C room temperature (RT), 60% humidity, lights on from 08.00 to 20.00 h) with food and water available ad libitum. The 12 and 24 month-old rats were weighed regularly in order to monitor their food intake, and tested in an open field setting for possible age-related differences in behavioral reactivity. No obvious abnormalities or overt age-related deficits could be observed (data not shown). The local animal ethical committee of the University of Amsterdam approved of all experiments. Animals were injected in the morning with BrdU (i.p., 10 mg/ml dissolved in 0.007N NaOH/0.9% NaCl) at a dose of 50 mg/kg for three times a day with 2 hours intervals and studied at 24 hours survival time after the first injection. Following perfusion fixation and in situ post-fixation 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 30 μm thickness using a sliding microtome. Sections were stored at 4°C in 0.1 M phosphate buffer, pH 7.4, with 0.01% azide until
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Reca / Ki-67 double immunocytochemistry

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 endogenous proliferation marker Ki-67.

**Day 1:**

Free-floating sections were washed in 0.1 M 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 powder (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 (Serotec, 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 (NGS) / 0.3% Triton X-100 in TBS (TBS+) was added to block nonspecific binding. Incubation of the 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., 2004a). Briefly, mounted tissue sections were pretreated in a domestic microwave 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 powder 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% H₂O₂ and 0.04% Nickel for 6-10 min, after which sections were washed, dehydrated, passed through xylene and coverslipped with Entellan.

**BrdU, Ki-67 and Reca immunofluorescence.**

Co-localization of BrdU and Ki-67 near Reca-positive blood vessels was examined in the subgranular zone (SGZ) and hilar region. To denature DNA, sections were incubated for 30 min in 2N HCl at 37°C, and neutralized for 10 min in 0.1 M boric acid, pH 8.5. After several rinses in TBS pH 7.6, and 30 min of incubation in 2% milk in TBS (TBS+), sections were incubated with the primary antibody rat anti-BrdU (1:2000, Accurate Chemical, Westbury, NY), mouse anti-Reca (1:250, Serotec, Oxford, UK) and rabbit anti-Ki-67 (1:50, Novocastra Laboratories, New Castle, UK) diluted in TBS / 0.25% gelatine / 0.5% Triton X-100 for 1 hr at RT and then 48 hrs at 4°C. After several rinses in TBS and incubation in TBS+ for 30 min, the first antibodies were subsequently detected with Alexa Fluor 546, Alexa Fluor 488, and Cy5, respectively and embedded in Vectashield (Vector Laboratories).

**Immunocytochemistry p27Kip1**

Free-floating sections were washed in 0.01M phosphate buffered saline (PBS) pH 7.4 and placed in plastic jars filled with citrate buffer (0.01 M, pH 6.0) and placed in a microwave oven for 10 min at 400W. After 30 min cooling at room temperature, endogenous peroxidase activity was blocked by 1.5% peroxide treatment for 15 min. After several rinses in PBS, 5% NGS / 0.3% Triton X-100 / 1% BSA in PBS was applied for 1 hr in order to prevent nonspecific binding. Sections were incubated with the primary antibody mouse monoclonal anti-p27Kip1 (K25025; 1:3000; BD Transduction Laboratories) diluted in 1% NGS / 0.3% Triton X-100 / 0.1% BSA in PBS (PBS+), for 1 hr at RT and then overnight at 4°C. Specificity of these antibodies has been demonstrated before elsewhere. For negative control, the first antibody was omitted. With intermittent rinses in PBS, sections were then incubated with biotinylated sheep anti-mouse IgG (1:200) (Amersham Life Sciences, Den Bosch, Netherlands, 1:200) in PBS+ / TBS+ for 1.5 hrs and amplified with ABC (1:800) in
PBS / BSA 1% for 2 hrs. The ABC signal was further amplified with biotinylated tyramide (1:500) and 0.01% peroxide in PBS for 30 min followed by another 1.5 hr incubation in ABC (1:1000). Color development was performed with nickel-enhanced diaminobenzidine (0.50 mg/ml DAB / 0.04% nickel ammonium sulphate / 0.01% H₂O₂) after which sections were mounted, dried, dehydrated, passed through xylene and coverslipped with Entallan (Merck).

Quantification and stereology

Serial sections (30 μm, every 10th 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 sections were photographed with a Nikon Coolpix 4500 digital camera connected to a Zeiss Axiophot microscope (10× objective). In view of possible anatomical differences along the septotemporal axis (Grivas et al., 2003), this was quantified in a stereological approach (every 10th). 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.

Immunofluorescent triple labeled sections 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. BrdU-positive cells were categorized in 3 groups according
to their association with the vasculature, colabeling with Ki-67 and association with Ki-67-positive cell clusters. A cell cluster was defined as a group of cells consisting of 2 or more cells. Colocalization of BrdU with Ki-67 immunoreactivity was determined after visual inspection of the XY, YZ, and XZ views. Since the numbers of BrdU-positive cells in the 12 and 24 month-old rats were very low and hardly any BrdU / Ki-67 double labeled cells were seen, statistically meaningful numbers could only be obtained from the 2 and 6 week-old animals.

Statistics

Statistical analysis was performed using analysis of the variance (ANOVA) and / or an unpaired Student's t-test with a two-tailed p-value when appropriate. 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

We investigated whether aging influenced the proportion of adult-generated cells associated with the vasculature (Figure 1A, see Appendix). The proportion of VA cells, expressed as a percentage of all Ki-67 positive cells in the SGZ and the hilar region (Figure 2) decreased during aging, although not significantly (2w->24m: ANOVA; F = 2.35, p = 0.09). However, between 6 week- and 12 month-old animals the VA proliferation decreased significantly (Mann Whitney test; p = 0.01).

Figure 2: VA proliferation decreases during aging. The proportions of VA proliferating cells are expressed (+/- SEM) as percentages of all the Ki-67-positive cells in the DG (= SGZ + hilus) of the 2 week-, 6 week-, 12 month- and 24 month-old rats. VA proliferation decreased during aging, however, only significantly between 6 weeks and 12 months of age (Mann Whitney test; = 0.01).
Since we previously reported that the numbers of newborn cells decreased differentially during aging in the different subregions of the dentate gyrus (Heine et al., 2004a), we additionally expressed the proportions of NA and VA proliferating cells found in the SGZ and hilus, as percentage of all the Ki-67 positive cells, as shown in Figure 3. During aging, the proportion of NA and VA proliferating cells in the SGZ decreased significantly (SGZ NA: ANOVA, \( F = 21.9, p < 0.0001 \); SGZ VA: ANOVA, \( F = 22.1, p < 0.0001 \)). By contrast, the proportion of proliferating cells in the hilus increased significantly. However, in the hilus, the fraction of NA proliferating cells increased much more (hilus NA; ANOVA, \( F = 35.9, p < 0.0001 \)) than the fraction of VA proliferating cells (hilus VA; ANOVA, \( F = 10.7, p < 0.0001 \)). So, more than 50% of the proliferating cells in the 24 month-old rats reside in the hilus, and is not associated with the vasculature.

**Figure 3:** In old age proportional more proliferating cells reside in the hilus that are not associated with the vasculature.

The proportions of VA and NA proliferating cells are expressed (+/- SEM) separately for the DG subregions: SGZ and the hilus, as percentages of all Ki-67-positive cell measured in both regions (SGZ NA + SGZ VA + hilus NA + hilus VA = 100%). During aging the proportion of NA and VA proliferating cells significantly decreased in the SGZ (SGZ NA: ANOVA, \( F = 21.9, p < 0.0001 \); SGZ VA: ANOVA, \( F = 22.1, p < 0.0001 \)), and significantly increased in the hilus (hilus NA; ANOVA, \( F = 35.9, p < 0.0001 \); hilus VA; ANOVA, \( F = 10.7, p < 0.0001 \)).

To address whether aging had affected the vascular bed itself and thereby, possibly indirectly, influenced VA proliferation, the surface area covered by the microvasculature was measured in Reca immunolabeled sections (Figure 1B, see Appendix). Results are presented in Figure 4. The proportion of surface area covered by the vasculature changed during aging (2w->24m: ANOVA, \( F = 8.8, p = 0.0005 \)). Following an initial reduction between 2 and 6 weeks of age (Mann Whitney test; \( p = 0.03 \)), the surface area covered by the vasculature significantly increased from 6 weeks of age onwards into old age (24 m) (ANOVA, \( F = 42.9, p < 0.0001 \)).

**Figure 4:** The proportion of surface area covered by the vasculature changes during aging.

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Figure 4:

Following an initial significant reduction between 2 and 6 weeks of age (Mann Whitney test; $p = 0.03$), from 6 weeks of age on into old age (24 m) the surface area covered by the vasculature significantly increased (ANOVA, $F = 42.9$, $p < 0.0001$).

To explore possible changes in the proliferative activity of newborn cells during aging, and assess whether or not this involved vascular association, sections were triple labeled for BrdU, Ki-67 and Reca (illustration in Figure 1C-F, see Appendix). BrdU-positive cells (24 hrs survival) were scored for colabeling with Ki-67, its association with a cluster of Ki-67-positive cells, as well as its association with a blood vessel (Reca labeling). Double labeled Ki-67 / BrdU cells were considered to have been in S phase 24 hrs ago, while still being in the cell cycle at present. A BrdU-positive cell near a Ki-67-positive cell cluster was considered to be an actively proliferating newly generated cell. The numbers of BrdU-positive cells were scored for: single or double labeling with Ki-67; association with a Ki-67 cell cluster; location in the SGZ or hilus; VA or NA. Results are expressed as percentage of all the Ki-67 positive cells in Figure 5.

In Figure 5A, the proportion of BrdU-positive cells associated with the vasculature is presented. Between 2 and 6 weeks of age, double labeling between BrdU and Ki-67 increased significantly ($p < 0.001$, t-test). Within the population of double labeled cells in the 6 week-old animals, especially the proportion of BrdU / Ki-67 cells proliferating near a cluster of Ki-67 positive cells increased significantly ($p = 0.0004$, t-test), while also the remaining population of BrdU / Ki-67 positive cells increased albeit to a lesser extent ($p < 0.002$, F-test). The population of single BrdU-positive cells decreased, but this was only significant for the cells located near a Ki-67 cell cluster ($p < 0.04$, Mann Whitney test).

In Figure 5B, NA BrdU-positive cell numbers are presented. Also in this population, the proportion of BrdU / Ki-67 doubled cells increased between 2 and 6 week-old animals ($p = 0.0025$, t-test); which was significant for the double labeled cells not residing near other Ki-67 positive cells ($p < 0.04$, Mann Whitney test). The proportion of single BrdU-positive cells decreased significantly between 2 and 6 week-old animals ($p = 0.01$, t-test).
Figure 5: The 6 week-old animals show significantly more actively cycling clusters of cells in the NA, but even more so in the VA population of proliferating cells, than the 2 week-old animals.

A) Within the population of double labeled cells in the 6 week-old animals, especially the proportion of BrdU / Ki-67 cells proliferating near a cluster of Ki-67 positive cells increased significantly (p = 0.0004, t-test), while also the remaining population of BrdU / Ki-67 positive cells increased (p < 0.002, F-test). The population of single BrdU-positive cells decreased. This was only significant for the cells present near a Ki-67 cell cluster (p < 0.04, Mann Whitney test).

B) Within the population of NA proliferating cells, the proportion of BrdU / Ki-67 doubled cells increased between 2 and 6 week-old animals (p = 0.0025, t-test); which was significant for the double labeled cells not residing near other Ki-67 positive cells (p < 0.04, Mann Whitney test). The proportion of single BrdU-positive cells decreased significantly between 2 and 6 week-old animals (p = 0.01, t-test)

To address whether aging affects the population of progenitor cells present in the DG, sections were immunocytochemically labeled for p27Kip1, a cell cycle inhibitor, low in post-mitotic neurons, and considered a marker for the progenitor cell population. In the 2 week-old animals, large numbers of positive cells were present in the SGZ, which decreases profoundly during aging with considerably less but still considerable numbers remaining even in old animals. Typical examples of p27Kip1-immunocytochemistry are shown in Figure 6.

Figure 6: Typical examples of p27Kip1-immunocytochemistry in 2 week-, 6 week-, 12 month-
and 24 month-old rat. A clear decrease is apparent particularly in the subgranular zone.

**Figure 6:**

![Image of vasculature in different age groups: 2 weeks, 6 weeks, 12 months, 24 months.](image)

**Discussion**

In this study, we show that aging affects the proportion of vascular-associated proliferating cells, notably in a regionally dependent fashion. Although the proportions of NA and VA proliferating cells in the SGZ decreased in a comparable manner, the proportion of NA proliferating cells in the hilus increased much more than the VA proliferating cells, with the NA population even making up over 50% of all proliferating cells in the 24 month-old animals. These differential changes were not due to a decrease in the vascular bed, as the surface area of the blood vessels rather increased between the ages of 6 weeks and 24 months. Neither are these changes likely to be caused by possible hippocampal shrinkage, since both DG volume and cell number did not change significantly between 6 weeks and 24 months of age in these very same animals (Heine et al., 2004a). The 6 week-old animals, with the lowest vascular surface area, nevertheless showed the highest VA proliferation. Furthermore, as shown by double labeling studies, this age group showed very active cycling clusters of new cells, especially near the vasculature. Since a major part of these proliferating cells will become mature granule cells (Heine et al., 2004a),
this supports an important role of the vasculature in the generation of new dentate granule cells.

In the older animals mainly "silent", single BrdU-labeled, non-vascular associated, newborn cells were present, while also the number of p27Kip1-positive cells in the dentate gyrus decreased, indicating a decline in the progenitor cell population as well. So, the age-related decline in neurogenesis is likely to occur due to alterations in the local microenvironment, such as in the microvasculature, as well as due to changes in intrinsic properties in the progenitor cell population.

In our hands the surface area covered by the blood vessels, a measure for the vascular bed, increased between the age of 6 weeks and 24 months. Many studies report age-related morphological changes in the brain vasculature (Goldman et al., 1987; Berman et al., 1988; Topple et al., 1990; Topple et al., 1991; Kalaria, 1996; Keuker et al., 2000), which include e.g. thickening of the basal lamina (Hicks et al., 1983; Topple et al., 1990). It is so far unclear whether the vascular bed is increased, decreased or unchanged during aging (review see Riddle et al., 2003). Although different results may partly be due to different brain regions that will respond differently during aging in this respect, conflicting results were reached even from studies involving the same regions in the same species. Amenta et al (1995), also studied male Wistar rats, but rather described an age-related decrease in vessel density in the hippocampus (Amenta et al., 1995). However, they used a completely different methodology on animals of different ages. Furthermore, differences along the septotemporal axis have been found in the vascular bed within the dentate gyrus, while also some small regional differences between the SGZ and hilus have been observed (Grivas et al., 2003). In our study, we measured the total surface area, including the SGZ and the hilar region, and hence cannot account for such regional changes. We conclude that at least the decrease in VA proliferation with age is not due to a decrease in structure of the vascular bed. Therefore, changes in the vascular integrity or other vascular-related factors, are likely to play a role in the age-related decline in proliferation.

We further showed that for both the 2 and 6 week-old animals, VA proliferating cells are generally closer to a cluster of active cycling (Ki-67-positive) cells than NA proliferating cells. This implies that locally produced vascular-related factors, e.g. neurotrophic factors, may stimulate proliferation, and keep adult generated cells in cell cycle. If so, it would then be reasonable to assume, that an age-impaired vasculature (including reduced levels of neurotrophic factors) might result in less VA proliferation during aging. For example, Yagita et al (2001) showed, that after transient forebrain ischemia, BrdU-positive cells have a higher survival rate in young rats, suggesting that
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despite a severe insult like ischaemia, younger brains have a more supportive environment for survival (Yagita et al., 2002). Consistent with this, Zaman and Shetty (2002) have shown that application of neurotrophic factors, such as BDNF and NT-3, increase the survival of grafted fetal hippocampal cells into old rats (Zaman & Shetty, 2002). Furthermore, IGF-1 is one of these endothelial-derived neurotrophic factors. IGF-1 is positively correlated with hippocampal neurogenesis (Anderson et al., 2002), has been shown to decrease during aging, while, interestingly, IGF-1 serum levels have been proposed to correlate with cognitive performance (Aleman et al., 1999; Busiguina et al., 2000; Lichtenwalner et al., 2001). Also Nakatomi et al. (2002) when studying endogeneous precursor proliferation following (ischaemic) damage, observed that intraventricular infusion of growth factors markedly augmented the proliferative responses, thereby increasing the number of newborn neurons, which significantly improved structural as well as functional recovery.

The importance of endothelial cells in the neurogenic niche is further supported by a study of Fukunaga et al (1999), who showed that CNS stem cells that were implanted into ischemic rat brains could differentiate and develop into mature CNS tissue. Notably, this consisted of vessels in and around neuronal graft cells (Fukunaga et al., 1999), further suggesting that the new cells in the DG might indeed play a role in angiogenesis. Moreover, a recent study reported on a decreased angiogenic response during aging, which occurred notably in close parallel to a reduced endogenous VEGF production (Wang et al., 2004). Adenoviral-VEGF121 gene transfer effectively augmented angiogenesis, particularly in the older animals. In conclusion, although age-related decreases in neurotrophic factor expression clearly affect new cell proliferation in the dentate gyrus, particularly VEGF, that was shown to have neurogenic and neuroprotective actions (Jin et al., 2002; Louissaint et al., 2002; Fabel et al., 2003; Heine et al., 2004c), appears very relevant with respect to its age-related decrease that might also contribute to the decline in hippocampal neurogenesis.

To obtain more insight in the proliferative activity of the newborn cells, and to compare those that are close to or more distant from the vasculature, confocal double labeling was performed. Cells double labeled for Ki-67 and BrdU, were considered to have been in S phase 24 hrs ago, while still being in the cell cycle at present. Considering the BrdU survival time of 24 hours, another explanation is that double positive cells have reentered the cell cycle for another cell division. In the middle-aged and old rats, however, the incidence of newborn cells was very low (Heine et al., 2004a). Moreover, while most cells present in these 2 groups were only single and solely BrdU-positive, only proliferating clusters in the 2 and 6 week-old rats were studied in more detail.
In the 6 week-old animals, many more double BrdU / Ki-67-positive cells were found than in the 2 week-old animals, for both the NA and VA proliferating cells. This suggests that more proliferating cells in the 6 week-old animals had reentered the cell cycle. Furthermore, in 2 week-old animals, a larger proportion of new cells had become postmitotic and/or quiescent. However, quiescence is not very likely at this very young age and 24 hours after birth, so most of these cells are likely expected to engage in another cell cycle or are expected to differentiate in a glial or neuronal phenotype soon. Furthermore, in the 6 week-old animals most new cells are proliferating in actively cycling clusters in the SGZ, of which a major proportion was shown before to become mature granule cells (Heine et al., 2004a). At the age of 2 weeks, many proliferating cells are located in the hilus, most of which become glia in this region (Heine et al., 2004a). If proliferating glia cells would have a different cycling behavior, we predict that they might become post-mitotic more easily, and proliferate to a lesser extent in active cycling clusters than proliferating cells destined to become new granule neurons. Clearly, this awaits further research.

Whether the age-related decline in neurogenesis occurs due to alterations in the local microenvironment, or whether these changes are the result of intrinsic properties in the progenitor cell population, is presently unresolved in the literature (Zitnik & Martin, 2002) and difficult to study with the current methodologies. So far, it has not been possible to quantitatively assess the neural stem cell populations in young and old animals, because of the inability to distinguish the stem cells from their rapidly dividing progeny in situ. Previous studies addressing decreases in proliferation in the aging dentate gyrus generally used BrdU (Kuhn et al., 1996; Kempermann et al., 1998) and/or polysialylated neural cell adhesion molecule (PSA-NCAM) immunocytochemistry (Seki & Ara., 1995). In this study we preferred the (combined) use of the endogenous proliferation marker Ki-67, since it is present during all phases of the cell cycle (Endl & Gerdes, 2000) and allows cycling clusters of cells to be more readily visualized. Furthermore, the combination of Ki-67 with BrdU gives more insight in the temporal dynamics and origin of the clusters of proliferating cells. Double labeled cells were considered to have reentered the cell cycle for another cell division. However, if the hippocampal progenitor cells have an age-related change in cell cycle length, then this would be expected to influence BrdU / Ki-67 double labelling as well. In old mice, indeed such lengthening of the cell cycle has been shown to occur in the forebrain subependyma (Tropepe et al., 1997). Consequently, changes found in the numbers of double labeled cells, most likely indicate age-related changes in the cycling behavior of the newborn cells in the DG.

Another aspect is the size of the progenitor population that may change with
A recent study showed that the neural stem cell population in the SVZ, i.e. not the SGZ, decreased two-fold in old mice. Subsequent analysis of neurosphere recovery in culture using a clonogenic assay, confirmed the factor two decrease between young and old animals. Since it is difficult to tell them apart, this observation reflects decreases in both the NSC as well as proliferative progenitors (Maslov et al., 2004). The authors conclude that the declining neurogenesis with age in the SVZ is a consequence of the loss of slowly cycling NSCs. However, in this study, not much is known about the influence of parallel changes in (micro)environmental factors that, based on the differential anatomy, are not necessarily comparable in the SVZ and hippocampal SGZ. It is attractive to speculate that also in the SGZ, the age-related decline in neurogenesis may, at least in terms of the number of neural stem cells, be intrinsically programmed.

The latter view is supported by the presently observed age-related decrease in the p27Kip1 cell population in the SGZ (Figure 6). P27Kip1 can inhibit activity of cyclins and their partner Cdk's, and so block cell cycle progression (Polyak et al., 1994). Van Lookeren Campagne and Gill (1998) studied the spatial and temporal expression of p27Kip1 in the developing central nervous system of the rat and showed that p27Kip1 mRNA expression was high in the proliferating ventricular and subventricular zones from E16 to E20, yet very low in the cortical plate, a densely packed zone of mainly postmitotic cells. Furthermore, p27Kip1 expression appears high in the proliferating cell populations residing in the postnatal lateral ventricles and is further involved in the regulation of cell proliferation throughout adulthood (van Lookeren Campagne & Gill, 1998; Doetsch et al., 2002). As such, p27 can be interpreted as a progenitor marker. In addition to the SVZ, our current observations of a prominent decrease in the number of p27Kip1 expressing cells in older animals, suggests an intrinsic, age-related decrease in the progenitor population, also in the SGZ.

Together with various reports our results suggest now indicate that both environmental as well as intrinsic factors within the progenitor population of the SGZ are responsible for the age-related decline in hippocampal proliferation (Hodges et al., 2000; Kempermann et al., 2002; Zitnik & Martin, 2002; Maslov et al., 2004). A better understanding of these both factors is essential for possible future brain repair approaches using transplantation of healthy progenitor cells into the old or injured brain.
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