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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Chapter 3

Prominent Decline of Newborn Cell Proliferation, Differentiation and Apoptosis in the Aging Dentate Gyrus, in Absence of an Age-Related Hypothalamus-Pituitary-Adrenal Axis Activation

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Neurobiology of Aging 2004 Mar;25(3):361-75
Abstract

Neurogenesis and apoptosis in the hippocampal dentate gyrus occur during development and adulthood. However, little is known about how these two processes relate to each other during aging. In this study we examined apoptosis, proliferation, migration and survival of newborn cells in the young (2 weeks), young-adult (6 weeks), middle-aged (12 months) and old (24 months) rat dentate gyrus. We also measured dentate volume and cell numbers, along with basal corticosterone and stress response parameters. We show that new cell proliferation and apoptosis slow down profoundly over this time period. Moreover, migration and differentiation into a neuronal or glial phenotype was strongly reduced from 6 weeks of age onwards; it was hardly present in middle aged and old rats as confirmed by confocal analysis. Surprisingly, we found no correlation between cell birth and corticosterone levels or stress response parameters in any age group.
Introduction

Both cell birth and apoptotic cell death are common during central nervous system development. Decades after its initial description (Altman & Das, 1965), it is now widely accepted that neurogenesis continues to occur also in the adult brain in the subventricular zone (SVZ) and the hippocampal dentate gyrus (DG) (Altman & Bayer, 1990; Eriksson et al., 1998; Gage, 2002).

Cell birth and cell death appear closely associated in the DG as a continuous cell turnover takes place. Consequently, the DG consists of a diverse and heterogeneous group of mature and developing cells. This turnover is furthermore highly sensitive to various hormonal and environmental stimuli (Gould et al., 1999b; van Praag et al., 1999b; van Praag et al., 1999a; Eisch et al., 2000; Gould et al., 2000). Removal of steroid hormones by adrenalectomy (ADX) e.g., induces apoptosis in the DG, but at the same time increases the division of immature cells (Cameron & Gould, 1996). On the other hand, stress reduces new cell birth (Fuchs & Flugge, 1998; Gould et al., 1998; Gould et al., 1999b; Gould et al., 1999a). In contrast, an enriched environment or learning tasks stimulate neurogenesis (Kempermann et al., 1998b; Kempermann et al., 1998a; Gould et al., 1999b; Gould et al., 1999a). The influence of age on rates of neurogenesis and apoptosis has not been studied in detail. Changes in the relative proportion of young DG neurons may have considerable consequences for hippocampal function and could possibly contribute to age-dependent structural and functional hippocampal deficits (Bondareff & Geinisman, 1976; West et al., 1991; Barnes, 1994; Bhatnagar et al., 1997). Moreover, relatively little is known about the life span of the individual newborn cells as the organism ages.

In the present study, we examined the hypothesis that age of the animal influences 1) the balance between neurogenesis and apoptosis, and 2) the process of migration and neuronal maturation of individual cells. To test this, we stereologically assessed birth rate, migration and survival of the newborn cells, as well as cell death and structural maturation of the DG in young (2 weeks), young-adult (6 weeks), middle-aged (12 months) and old (24 months) rats. Dividing cells were labelled with bromodeoxyuridine (BrdU) and studied after survival times of 24 hours, 1 and 4 weeks. Triple immunocytochemical analysis (BrdU, NeuN and S100β) was used to study age-related changes in the phenotype of adult generated cells.

Elevated glucocorticoid (GC) levels are frequently found in aged rats and may contribute to age-related memory and learning deficits (Lupien et al., 1998; Porter & Landfield, 1998; Hibberd et al., 2000; McEwen, 2000; Lucassen et al., 2001). In addition,
stress and corticosterone a) can reduce adult progenitor proliferation and b) may relate to the decline in neurogenesis during aging. Following ADX, neurogenesis indeed increased in old rodents, suggesting that reduced neurogenesis during aging (Kuhn et al., 1996; Kempermann et al., 1998b) might be causally related to increased GC levels (Cameron & McKay, 1999; Montaron et al., 1999). Therefore, basal corticosterone levels and the corticosterone response to a mild stressor (indicators of hypothalamus-pituitary-adrenal (HPA) axis activity and feedback function, respectively) were correlated with age-dependent changes in dentate cell turnover.

**Material & Methods**

**Animals**

Middle aged and old male Wistar rats were obtained from the aging colony of the Institute of Physiological Psychology, University of Düsseldorf, Germany. Animals were studied at 6 weeks (young adults) (n = 9), 12 months (middle aged) (n = 10) and 24 months of age (old) (n = 10). Since neurogenesis is expected to be maximal during the first two weeks of postnatal life (Schlessinger et al., 1975), also 2-weeks-old male Wistar rats were included (n = 10), which were housed with five animals and one mother in a cage. Six-weeks-old animals were chosen, for at this age the effects of glucocorticoid manipulation on e.g. hippocampal function or structure are still considerable (Stienstra et al., 1998; Karst & Joels, 2001; Wossink et al., 2001). The middle-aged group was included in view of possible midlife changes (Coleman, 1989; Coleman et al., 1990). The 24-months-old group was chosen because it fits the criteria of old age for a Wistar rat.

After transport to our facilities, the animals were first left undisturbed for two weeks before experiments commenced. The animals were single housed under controlled conditions (21°C room temperature, 60% humidity, lights on from 8.00-20.00h) 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 in order to investigate possible age-related differences in behavioral reactivity. No obvious abnormalities or overt age related deficits could be observed. So these data are not presented.

In order to visualize newborn cells, the 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 50 mg/kg for 3 times a day with 2-hour intervals. They were studied at 24 hours, 1 week, or 4 weeks survival time (S) after the first injection in order to study proliferation rate, migration, and differentiation. The local animal ethical committee by the University of Amsterdam
approved all experiments.

**Stress response**

In order to determine basal corticosterone levels and the response to a mild stressor, naive animals of the 6w-, 12m- and 24m-age-group were placed in a novel cage after which blood samples were drawn at 0, 15, 30, 60 and 120 min by tail incision (Durschlag et al., 1996). All efforts were made, also during housing, to prevent our animals from over handling, or from treating them in any other way that could have masked their HPA activation. Blood was centrifuged for 20 min at 5000 rpm at +4°C and then stored at -20°C until corticosterone concentration was determined using radio immunoassay (ICN Biochemicals Inc.)

**Brain tissue preparation**

Animals were deeply anesthetized 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 intracardially with 0.9% physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. After *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 needed.

**Antibodies**

The following antibodies and final dilutions were used: mouse anti-BrdU (1:3000, Roche Diagnostics, The Netherlands), rat anti-BrdU (1:50, Accurate Chemicals, Westbury, NY), mouse anti-NeuN (neuronal marker) (1:500, Chemicon, Harrow, UK), rabbit anti-S100β (glial marker) (1:1000, Swant, Bellinzona, Switzerland), polyclonal rabbit anti-Ki-67 (1:2000, Novocastra, New Castle, UK), biotinylated sheep anti-mouse IgG (1:200, Amersham Life Sciences, Den Bosch, The Netherlands), biotinylated sheep anti-rabbit IgG (1:200, Amersham Life Sciences, Den Bosch, The Netherlands), avidin-biotin-peroxidase complex (1:1000, Vectastain Elite, Brunschwig Chemie, Amsterdam, The Netherlands), goat anti-rat-Alexa Fluor 546, goat anti-mouse-Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) and goat anti-rabbit-Cy5 (1:200, Jackson, The Netherlands)
**BrdU immunoperoxidase**

To detect BrdU-labeled nuclei, free-floating sections were treated with 1% $H_2O_2$ in 0.1 M phosphate buffer (PB) for 30 min to block endogenous peroxidase. To denature DNA, sections were incubated for 2 hrs in 50% / 2xSSC (0.3 M sodium chloride and 0.03 M sodium citrate) at 65°C, incubated for 30 min in 2 N HCl at 37°C, and neutralized for 10 min in 0.1 M boric acid, pH 8.5. After several rinses in PB, 1 hr of incubation in PB / 0.1% bovine serum albumin (BSA) / 0.3% Triton X-100 / 1% goat serum (PB+) followed. Sections were incubated with the primary antibody mouse anti-BrdU diluted in PB+ for 1 hr at RT and then overnight at 4°C. With intermittent rinses in PB, sections were incubated with biotinylated sheep anti-mouse IgG for 2 hrs and avidin-biotin-peroxidase for 2 hrs. Colour development was performed with diaminobenzidine (0.50 mg/ml DAB / 0.01% $H_2O_2$) for 30 min, after which sections were mounted, dehydrated, passed through xylene and coverslipped with Entallan (Merck).

**Ki-67 immunoperoxidase**

The Ki-67 antigen is a 345 to 395 KDa non-histone protein complex present only in proliferating cells during $G_1$, $S$, $G_2$ and $M$, but not the $G_0$ phase of the cell cycle (Gerdes et al., 1984; Endl & Gerdes, 2000). Furthermore, Ki-67 staining is highly comparable to BrdU counts after short survival times, and is a well-accepted proliferation marker in tumor biology (Gerdes et al., 1991; Kee et al., 2002). To detect Ki-67 antigen positive nuclei, free-floating sections were mounted onto plus glass slides (Menzel), dried overnight at 37°C, and rinsed with 0.1 M Tris buffered saline pH 7.6 (TBS). Sections were placed in plastic jars filled with citrate buffer (0.01 M, pH 6.0) and put in a microwave (MW) oven. For standardization purposes, two filled jars were always used, irrespective of the number of sections, and 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. After 30 min cooling at room temperature, endogenous peroxidase activity was blocked by 15 min 1.5% peroxide treatment. After several rinses in TBS, 2% milk powder (Elk, Campina Melkunie, Eindhoven, The Netherlands) solution was applied for 30 min in order to prevent nonspecific binding. Sections were incubated with the primary antibody anti-Ki-67 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 anti-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
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(1:500, produced and kindly provided by Dr. I. Huitinga, Netherlands Institute for Brain Research, Amsterdam, The Netherlands) and 0.01% peroxide in TBS for 30 min followed by another 1.5 hr incubation with ABC (1:1000). Color development was performed with diaminobenzidine (0.50 mg/ml DAB / 0.01% H$_2$O$_2$) for 6-10 min after which sections were dehydrated, passed through xylene and coverslipped with Entallan. Sections were lightly counterstained with haematoxyline.

TUNEL

Terminal transferase mediated dUTP nick-end-labeling (TUNEL) was performed to detect cells undergoing apoptosis as described earlier in detail (Lucassen et al., 1997; Lucassen et al., 2000; Lucassen et al., 2001) with minor modifications. Free-floating sections were mounted onto plus glass slides (Menzel), dried overnight at 37°C and rinsed with 0.01 M PBS (KH$_2$PO$_4$, Na$_2$HPO$_4$,H$_2$O) pH 7.2. Sections were then pretreated in 0.1 M sodium citrate buffer (pH 6.0) in a microwave oven, set at full power for 5 min. After cooling the jars, sections were pre-incubated with Proteinase K buffer (10 mM Tris / HCl, 2.6 mM CaCl$_2$, pH 7.6) for 10 min and then incubated with 20 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) 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 / 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, The 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% H$_2$O$_2$ 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 avidin-biotin-peroxidase in PBS / 1% BSA overnight at 4°C. Peroxidase detection (0.50 mg/ml DAB / 0.01% H$_2$O$_2$) was done for 1-2 min, based on positive control sections included in every assay. Sections were lightly counterstained with cresyl violet and quantified stereologically.

BrdU, NeuN and S100β immunofluorescence

Co-localization of neuronal marker NeuN, or glial marker S100β immunoreactivity with BrdU immunoreactivity was examined in the subgranular zone (SGZ) and granular cell layer (GCL). For DNA denaturalization, sections were pretreated as described above for BrdU. Afterwards sections were rinsed for one hour in 0.05 M TBS
and incubated in TBS / 1% BSA / 0.1% Triton X-100 / 3% goat serum (TBS++) for 1 hr and for 48 hrs at 4°C with the primary antibodies: rat anti-BrdU, mouse anti-NeuN, rabbit anti-S100β 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, Alexa Fluor 488 and Cy5, respectively and embedded in Vectashield (Vector Laboratories).

Quantification and stereology

Serial sections (30 μm, every 20th section) were taken for stereological quantification of dentate granule cell number and volume as well as for TUNEL- and BrdU-positive cells. Because newborn cells in the 12- and 24-months-old rats are in low abundance, every 10th section for these age groups was included in quantifying the number of BrdU-positive cells. The BrdU-positive cells were analyzed per hippocampal subregion (hilus, SGZ, GCL and molecular layer) to study migration of the new cells. The numbers were assessed 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 GCL of the dentate gyrus, 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 areas occupied by the GCL for the full rostral-to-caudal series were measured on a Macintosh computer, 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 volume of the GCL was calculated from \[ V = \sum A \times T \], where \( \sum A \) is the sum of area measurements and \( T \) is the intersection distance 600μm.

The same sections were used to determine the three-dimensional numerical density of neurons (\( N_v \), neurons per cubic millimeter) in the GCL, using the optical dissector method (West et al., 1991). Object-Image software was used to randomly place a square counting frame over the GCL on the reference section. Individual neurons were visualized using the Zeiss Axiophot microscope (100× NA 1.30 oil objective) and counted with the dissector 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 (\( N_v \)) of neurons from \[ N_v = \frac{Q}{\sum V_{\text{dis}}} \], where
\[ \Sigma Q \] is the sum of the neurons counted in all dissectors and \( \Sigma V_{\text{dis}} \) is the sum of the disector volumes. One disector volume is 17280 \( \mu \)m, 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 \( \mu \)m on a side, and \( h \) the disector height of 30 \( \mu \)m. The total number of neurons in the GCL was calculated from estimates of neuronal \( N_v \) and the total volume of the GCL.

Immunofluorescently stained sections were evaluated using a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal laser-scanning device equipped with a Plan-Neofluar 100\( \times \) / 1.3-oil lens. Co-localization of NeuN with BrdU immunoreactivity was determined after visual inspection of the XY, YZ and XZ view. The proportion of co-localization was calculated in the 12- and 24-months-old rats, based on the total number of double-labelled cells scored in every 20th section of each animal of these age groups.

**Statistics**

Statistical analysis was performed using ANOVA to test the standard deviation on equal distribution, followed by 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 nonparametric Mann-Whitney-U-test was applied to the data. Correlations were calculated using the two-tailed (parametric) Pearson’s test.

**Results**

Total volume and cell numbers of the DG were stereologically determined in Nissl-stained sections and grouped by age (Fig. 1). Both parameters showed an almost two-fold increase between the 2- and 6-week old animals. Although at 12 months rats showed a significant decrease in DG volume compared to 6 weeks \( (p = 0.014) \), neither of these two groups was significantly different from 24-month old rats. Total DG cell number remained stable from 6 weeks onwards. A correlation coefficient of \( r = 0.995 \) was present between DG volume and total cell number. Thus, after a pronounced increase in volume and cell numbers in the first few postnatal weeks, both parameters showed no further significant declines during aging.

*Figure 1:* DG volume and granule cell numbers increase at young age, but remain stable from 6 weeks of age onwards.

*(A):* Mean estimated volume (SEM) of the hippocampal dentate granular cell layer for 2-week, 6-week, 12-month and 24-month old Wistar rats.

*(B):* Mean estimated total neuron number (SEM) for 2-week, 6-week, 12-month and 24-month old Wistar rats.
Examples of BrdU-positive cells are shown in Figs 2, 3 and 4. Since no counterstaining was used, they were photographed under Nomarski optics to provide increased contrast and allow clear visualization of the structural borders of cell layers. The number of newborn, BrdU-positive cells was very high at 2 weeks and less so at 6 weeks, particularly when compared to middle-aged and old rats (Fig. 2A). Comparing the location of the BrdU-positive cell numbers per hippocampal sub-region (hilus, SGZ, GCL, molecular layer) revealed that newborn cells were present in all four regions in all age groups, with the hilus and SGZ outnumbering the other areas, particularly in young animals. Very low numbers of new cells were seen in 12- and 24-month old rats (Figs. 2A-E).

Ki-67 immunocytochemistry revealed isolated cells with clear nuclear staining occurring at low frequencies, mainly in the hilus and SGZ sub-region. Obvious doublets of cells were also observed representing dividing cells (Fig 2F, arrow, see Appendix). The numbers of Ki-67 positive cells were comparable to BrdU counts 24 hours after BrdU injection (i.e. ~2-4 cells per section) in 12- and 24-month old rats.

Figure 2: Proliferation of newborn cells during aging reveals a prominent reduction in middle-aged and old animals.

(A): Quantification of the amount of cell birth, expressed as the estimated mean total number old Wistar rats.

Average volume and granule cell number was statistically indistinguishable between 12 and 24 months of age. * p < 0.01 compared with 6w, 12m and 24m; ** p = 0.014 compared with 6w (Student's t test).
Figure 2A:

(Bar graph) of BrdU positive cells per region of the hippocampus 24 hours after BrdU injection in 2-week, 6-week, 12-month and 24-month old rats:

Typical examples of BrdU immunohistochemistry in a 2-week (B), 6-week (C), 12-month (D) and 24-month old rat. The sections are not counterstained. Therefore photographed under Nomarski optics, to increase contrast and to visualize better the structural borders of the cell layers. A typical example of Ki-67 immunocytochemistry in a 12-month rat (F, see Appendix) (bright field) shows comparable amounts of proliferating cells as shown by BrdU immunolabeling after 24 hrs survival.

Figure 2B, C, D, E:

B: 2 weeks

C: 6 weeks

D: 12 months

E: 24 months

Analysis of migration patterns (Figs. 3A and 3B) revealed that in 2-week old rats, 24 hours after BrdU injection, clusters of new cells were present in both hilus and SGZ (Fig. 3A3). At the same age, after one-week survival, BrdU positive cells had further increased in number and already lined up along the GCL border or even entered it (Fig.
At 6 weeks of age however, the number of new cells was already significantly reduced (Fig. 3B1 and 3B2). Almost all proliferating cells were still located mainly in the SGZ after 24 hours of survival (Fig. 3B3). BrdU-positive cells that were allowed to survive for one week in this age group (Fig. 3B4) were seen in all regions, but not that prominently in the GCL as with 2-week old rats after 1-week survival. In 6 weeks old rats, most BrdU-positive cells had arrived in the GCL (Fig. 3B5) after 4 weeks of survival. Notably, for this population, the BrdU injection had been administered at 2 weeks, and the absolute number of cells is therefore comparable with the number of proliferating cells in the DG of that age (i.e., 2-week-old / 24-hour-survival) (Fig. 4). Less than half the newborn cells at this particular age (2 weeks) actually survived for 4 weeks (Fig. 4); total number of cells after 4 weeks was significantly reduced ($p = 0.0038$). The numbers of migrating cells in middle-aged and old rats declined to very low numbers (Fig. 3C and D), and no differences were seen between 12- and 24-month old rats in proliferation or in migration over a four-week survival period (Fig 3C3 and 3D3). Following the rapid increase in new cells in the two-week old rats due to further division and rapid migration, both proliferation as well as migration slowed down to very low levels starting from 6 weeks of age.

**Figure 3:**

3A1: 2 weeks

3A2: 2 weeks

3A3: 2 weeks - 24 hours S

3A4: 2 weeks - 1 week S
Migration of newborn cells into GCL is slowed down strongly between 2 and 6 weeks of age and rarely observed in 12- and 24-month rats. Asterisk indicates a statistically significant increase in the amount of BrdU-positive cells compared to the shorter survival population of new cells in that area within the same age group (p < 0.001, Student's t test).

Representative examples of BrdU immunohistochemistry are shown in a 2-weeks old rat, 24 hours (3A3) and 1 week (3A4) after BrdU, clear migration into the GCL is observed; Fig. 3B shows a 6-week old rat, 24 hours (3B3), 1 week (3B4) and 4 weeks (3B5) after BrdU injection; clear migration into the GCL is accompanied by morphological alterations in the nuclear pattern of BrdU staining (arrow). Fig. 3C + D display a 12-month (3C3) and 24-month old rat, 4 weeks (3D3) after BrdU injection. The extensive migration of the newborn cells into the GCL seen in young animals hardly occurs anymore. At 4 weeks survival after BrdU, only very few cells are found which further appear to have divided only recently (arrow).
For phenotypic analysis of newborn BrdU-positive cells, tissue was immunocytochemically triple-stained for BrdU, the neuron-specific marker NeuN and glial marker S100β. Double-labeled images were inspected in the orthogonal planes X, Y and Z to verify double labeling throughout their extent (Fig. 5C, see Appendix). This revealed that in 2-week old rats, BrdU positive cells that had survived for one week (Fig. 5A, see Appendix) already showed double labeling with S100β (Fig. 5B, see Appendix) and NeuN (Fig. 5C, see Appendix). Although migration towards the GCL was apparent, no such double labeling was yet seen in the 6-week old rats one week after BrdU injection (Fig. 5E, Fig. 5D, see Appendix). In the 6-week old rats, NeuN-BrdU double positive cells were not seen until after 4 weeks of survival. Even after 4 weeks of BrdU survival, 12- and 24-month-old rats showed mostly undifferentiated
BrdU positive cells (Fig. 5F, Fig. 5G, see Appendix). Only a small percentage of these already small numbers of newborn cells in the DG showed NeuN-BrdU double labeling (14% in middle-aged and 7% in old rats). So, with aging, not only migration but also differentiation of individual new cells into mature granule cell appears to slow down. Only a very small fraction of these cells is fully differentiated after 4 weeks survival (Fig. 5H, see Appendix).

Figure 4: Less than half of the new cells born at 2 weeks of age survive for 4 weeks (animal age of 6 weeks).

Quantification of newborn and surviving cells, expressed as the estimated mean total number (+/- SEM) of BrdU-positive cells per sub-region of the hippocampus 24 hours after BrdU injection in 2-week old rats and 4 weeks after BrdU injection in 6-week old rats. Total numbers of new cells after 4 weeks S was significantly reduced compared to the 24 hrs S group (p = 0.0038). Most cells that survived for 4 weeks, ended up in the GCL i.e. 69% (see also Fig. 3B2).

As the DG is characterized by a constant turnover of granule cells, alterations in rates of apoptosis will affect the total population of granule cells. TUNEL-positive, dying cells, all displaying at least some of the main morphological hallmarks of apoptosis, were observed at low frequencies in all DG sub-regions and only very occasionally in areas outside the DG e.g. fimbriae and CA (not shown). A significant decline in the numbers of TUNEL-positive, apoptotic cells in the DG was seen between 2- and 6-week old animals (p < 0.001) (Fig. 6). From the age of 6 weeks onwards no further decrease was observed. A significantly higher amount of apoptotic cells was present within the SGZ of 6-week-old rats, compared to other sub-areas. Sub-areas in other age groups did not differ from each other. So changes in apoptosis play a major role in the youngest animals, but in contrast to proliferation, do not change much from early adulthood into middle age.

Figure 6: Spontaneous apoptotic cell death decreases during life span of rats.

(A): Numbers of apoptotic cells expressed as the estimated mean total number (+/- SEM) of TUNEL-positive cells per region of the hippocampus in 2-week, 6-week, 12-month and 24-month old rats. Asterisk indicates statistically significant difference between the SGZ and the other
B, see Appendix): Representative example of a TUNEL-positive, apoptotic cell in the hippocampal SGZ at the border with the DG-GCL. Inset shows a higher magnification of another example, displaying the main apoptotic hallmarks, such as obvious shrinkage and nuclear condensation, a brown DAB deposit indicating TUNEL identified DNA fragmentation and presence of clear apoptotic bodies.

In order to illustrate how ongoing birth and death in the DG relate to each other during aging, numbers of proliferating cells (survival time of 24 hours after BrdU) summated for the SGZ and GCL are presented parallel to the numbers of TUNEL-positive cells, expressed for the entire hemisphere (Fig. 7). Cell birth as well as death decline profoundly with age, though at different rates.

Figure 7: Cell birth and death in the dentate gyrus both decline during aging. Mean total number (+/- SEM) of BrdU- (24 hours after BrdU injection) and TUNEL-positive cells in the dentate gyrus of 2-week, 6-week, 12-month and 24-month old rats plotted to two different y-axis. Estimates are based on the sum of positive cells in the SGZ and GCL.

Basal corticosterone levels as well as the stress response were measured as indicator for HPA-axis activity and feedback function, respectively. Data are depicted in Fig. 8 and analyzed for statistical differences in a) basal corticosterone levels, b) peak height, i.e. the net corticosterone rise (peak minus basal value) independent of the time to peak and c) area under the curve (AUC) of the stress response over 120 min of the young adult,
middle aged and old animals. No significant differences were found in the basal levels between the age groups. The peak height was significantly larger in 6-week relative to 12-month old rats \((p = 0.015)\) signifying a rapid rise from relatively low basal levels to peak value. Contrary to our expectations, the AUC at 24 months was significantly smaller than at 6 weeks \((p = 0.001)\) and 12 months \((p = 0.034)\).

**Figure 8:**

![Graph showing corticosterone levels](image)

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Since 12-month rats showed the largest AUC value, perhaps indicating hypercorticism, total numbers of newborn cells per individual animal were tested for possible correlations with HPA-axis parameters. All 12-month old animals were included in this analysis i.e. those receiving BrdU injections 24 hrs, 1 week or 4 weeks earlier. No significant correlation could be found for AUC \((p = 0.46; \text{r}^2 = 0.07; \text{Pearson } r = -0.26)\) or basal corticosterone levels \((p = 0.58; \text{r}^2 = 0.04; \text{Pearson } r = -0.20)\). Also, correlations between numbers of BrdU cells in the SGZ + GCL +
hilus together, numbers of BrdU cells in SGZ + GCL alone, numbers of apoptotic cells, or total numbers of BrdU minus apoptotic cells failed to reach significance (not shown).

Table 1:

<table>
<thead>
<tr>
<th>Age</th>
<th>Basal Cort (µg/dl)</th>
<th>Cort rise&lt;sub&gt;net&lt;/sub&gt;</th>
<th>AUC</th>
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<tr>
<td>6w  (n=6)</td>
<td>6.6 ± 1.1</td>
<td>18.15 ± 1.25*</td>
<td>2195 ± 66 **</td>
</tr>
<tr>
<td>12m (n=10)</td>
<td>13.7 ± 2.7</td>
<td>11.01 ± 1.39</td>
<td>2360 ± 205 ***</td>
</tr>
<tr>
<td>24m (n=5)</td>
<td>5.7 ± 1.4</td>
<td>13.60 ± 2.51</td>
<td>1643 ± 96</td>
</tr>
</tbody>
</table>

Table 1: Basal corticosterone level and stress responses in rats of different ages. Mean (SEM) values of the basal corticosterone level, net corticosterone rise (peak minus basal value) and area under curve (AUC) of the 6-weeks, 12-months and 24-months old rats. Peak height was significantly larger in the 6-weeks old rats (*p = 0.015). AUC of the 24-month old rats was significantly smaller than in the 6-weeks (**p = 0.001) and 12-months old group (***p = 0.034).

Discussion

In the present study, we hypothesized that age would influence 1) the balance between neurogenesis and apoptosis in the rat hippocampus, as well as 2) the process of neuronal maturation. To test this hypothesis, we studied proliferation rate, migration and differentiation (and hence maturation) of the newborn cell, together with apoptosis in animals of different ages. As HPA axis activity was expected to change in an age-dependent manner and to influence proliferation and apoptosis, these changes were also studied in relation to HPA axis responsiveness.

In previous literature, various BrdU dosages, injection frequencies and survival times have been used to study adult neurogenesis (Seki & Araki, 1995; Gould et al., 1999b; van Praag et al., 1999b; Cameron & McKay, 2001; Rakic, 2002), each yielding different information concerning fate or numbers of newborn cells, making it difficult to compare these studies in terms of absolute numbers. Using our injection paradigm, we found that after the developmental peak of granule cell proliferation (i.e. 2 weeks of age), the more than 37,000 newborn cells (SGZ + hilus together, making up 9% of total granule cells) drop to approximately 6,000 newborn cells at 6 weeks of age (Figs. 2 and 3, less than 1% of total granule cells). From this age onwards, the rate of new cell birth declines dramatically to the very low numbers of proliferating cells found at 12- and 24-months (0.07% of total). Our Ki-67 immunocytochemistry revealed clear, isolated examples of proliferating and dividing cells in the same sub-regions where BrdU-positive profiles were also found after short survival time points (Fig. 2F, arrow / arrowhead). Numbers of Ki-67-positive cells
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were comparable to BrdU counts after 24 hrs of survival, which excludes the possibility of BrdU penetration or clearance differences in these age groups. It is also consistent with a recently published methodological paper that validated the use of Ki-67 signal to compare with BrdU numbers after short survival times (Kee et al., 2002).

With increasing survival time of the newborn cells (24 hrs, 1 week), fast migration of BrdU-positive cells into the GCL was apparent only in 2-week old rats; migration had already slowed down in young-adult rats (6 weeks) and was not seen in middle-aged and old animals. Moreover, of the very few BrdU-positive cells in the 24-month old rats, only a small fraction (i.e. 7%) had differentiated into a neuronal phenotype after 4 weeks survival (see above and Fig. 5H). Another possibility is that newborn cells could have divided further, diluting BrdU to undetectable amounts. However, in view of reduced migration, this does not seem very likely in old animals. Taken together, this demonstrates that aging strongly reduces proliferation and migration of newborn cells. The absence of a difference in DG number and volume between middle-aged and old groups is unlikely to be due to our methodology. Our stereological analysis involved the counting of over 100 cells, which is sufficient to achieve statistical power. Another confounding issue could be variations in section thickness. However, in view of the low variability in our 12- and 24-month old groups and the general agreement with literature, it is unlikely that this parameter has masked the existence of major differences between these 2 age groups. As also apoptotic rate was unchanged in middle and old age, our stereological quantification of structural, cell birth and death parameters shows that DG structure does not change much from young adulthood onwards, which is in line with other studies (Sousa et al., 1998).

This rapid slowing down of proliferation and migration during aging is intriguing as it suggests that the fate of newborn cells in the hippocampus is strongly influenced by local environmental factors rather than by intrinsic or genetic cues. Other studies, e.g. on heterotopic transplantation of harvested and clonally expanded precursor cells have also indicated that local environmental cues are involved in determination of the final, adult phenotype of the newborn cell (Shihabuddin et al., 2000). This may be related to age-dependent changes in the levels of growth factors or growth factor receptor expression in glia or neighboring neurons (Kuhn et al., 1997; Hansson et al., 2001). Not only does growth factor expression change with age in a differential manner, it is also influenced by stress hormones (Schaaf et al., 1999; Hansson et al., 2001) and related to age-related cognitive decline in performance (Schaaf et al., 2001). In addition, physical exercise through voluntary running increases hippocampal neurogenesis in mice (van Praag et al., 1999b; van Praag et al., 1999a), in addition to elevating growth factor expression and
neurotrophin levels in parallel (Neeper et al., 1995). In particular basic fibroblast growth factor (bFGF) is increased, which is known to be important for survival and differentiation of progenitor cells *in vitro* and *in vivo* (Kuhn et al., 1996; Kuhn et al., 1997). This neurotrophin is also known to regulate AMPA receptor subunit composition and Ca2+ responses in hippocampal neurons (Cheng et al., 1995). Although bFGF itself did not affect neurogenesis in adulthood (Wagner et al., 1999; Cheng et al., 2002), a study on age-related changes in hippocampal bFGF has, to our knowledge, not yet been performed. Another possibly relevant factor is polysialylated neural cell adhesion molecule (PSA-NCAM), that shows reduced immunoreactivity in aging and has been proposed to play a role in cell migration and differentiation (Kuhn et al., 1996; Abrous et al., 1997).

The peak in apoptosis in the SGZ at 6 weeks (Fig. 6A) suggests that at this age, many newborn cells die shortly after birth and close to their place of birth. Interestingly, our data shows that apoptosis decreases with age, although at a slightly different rate than proliferation, even though other studies reported increased (Lemaire et al., 2000) or stable (Cameron & McKay, 1999) numbers of dying cells with age. This suggests that the processes of birth and death are closely correlated which fits with the stability of DG cell number and volume with age. One explanation for the discrepancy between our observations and those made by Lemaire et al. (2000) could be that we only scored TUNEL-positive cells that show convincing morphological hallmarks of apoptosis. Lemaire et al. counted pyknotic cells in cresyl violet stained sections, which could have resulted in the inclusion of other profiles, e.g. glial cells or caps from cells in adjacent sections.

Consistent with other studies on young rats, the estimated numbers of newborn cells exceeds by far the numbers of dying cells (Biebl et al., 2000). This relates to the fundamentally different time kinetics of the two processes. The low frequency of apoptosis measurement in tissue sections is because of its short duration, i.e. hours, during which it can be detected (Bursch et al., 1990; Thomaidou et al., 1997; Conti et al., 1998; Clarke et al., 1999). Adrenalectomy-induced apoptotic cells in the rat DG were also only detectable for 72 hours at most (Hu et al., 1997). Hence, the chance of "trapping" ongoing apoptosis in thin tissue sections of a slowly progressing condition like age is very low (Lucassen et al., 1997; Perry et al., 1998b; Perry et al., 1998a). Over time, the contribution of apoptosis to structural DG changes could be considerable as it is in obvious balance with neurogenesis in this area; no changes were observed in DG cell number or volume between 12- and 24-month old rats.

The failure of 24-month old Wistar rats to show elevated basal corticosterone levels or increased stress responses was unexpected. In fact, the AUC of the stress response
of old animals was significantly smaller than in other groups. Aging is indeed associated with altered stress feedback sensitivity. Previous studies have often shown activation of the HPA axis or enhanced basal corticosterone levels in aged animals of different strains (Sapolsky et al., 1986; Brodish & Odio, 1989; Dellwo & Beauchene, 1990; Issa et al., 1990; Hauger et al., 1994; Seckl & Olsson, 1995; Sapolsky, 1999; Lucassen & De Kloet, 2001). However, exceptions also exist. Various reports failed to find elevated basal CORT levels or enhanced stress responses in old animals, and even found decreased glucocorticoid or ACTH levels in old animals (Sonntag et al., 1987; Issa et al., 1990; Scaccianoce et al., 1990; van Eekelen et al., 1991; van Eekelen et al., 1992; Morano et al., 1994; Cizza et al., 1995; Scaccianoce et al., 1995); for reviews, see (Seckl & Olsson, 1995; Lucassen & De Kloet, 2001). Notably, also in a well-accepted animal model to study aging, i.e. the Brown Norway rat, no indications for an age-related HPA axis activation could be found (van Eekelen et al., 1991; van Eekelen et al., 1992; Gomez et al., 1998; Workel et al., 2001). Hence, an obvious explanation for our contrasting results could be our use of the Wistar, rather than Fischer 344, Long-Evans or Sprague-Dawley rats, or relate to different HPA parameters studied, or differences in experimental design. HPA axis activation in rodent aging apparently occurs in many, but not all strains. In middle-aged and old Wistar rats, it is apparently not related to reduced proliferation seen in the hippocampus.

Others have suggested that adrenal steroid levels are inversely correlated with rate of cell proliferation in the dentate gyrus (for review see: Gould & Tanapat, 1999); two studies even reported that ADX at 24 months of age increased the proliferation rate up to levels found in young controls (Cameron & McKay, 1999; Montaron et al., 1999), suggesting that enhanced corticosterone levels might be responsible for reduced neurogenesis in old age.

Our current results do not support this notion. Not only were stress responses not different between different ages, neither were there obvious correlations between corticosterone levels and the 6-fold drop in numbers of BrdU positive cells between the 2- and 6-week animals. Very low numbers of newborn cells were seen in the 24-month group, where no elevated basal corticosterone values were found. Moreover, no significant correlations could be found for AUC or basal corticosterone levels and any of the categories of BrdU cell numbers (i.e. SGZ + GCL + hilus together, SGZ + GCL alone, numbers of apoptotic cells, or total BrdU minus apoptotic cells) in 12-month animals which showed at least some signs of hypercorticism (on average elevated basal levels, less efficient feedback and the largest AUC value). Our failure to find a relationship between proliferation and these stress parameters in either age group contradicts the hypothesis that
age-related declines in neurogenesis are related to increased basal corticosterone levels in old rats (Cameron & McKay, 1999).

As to the proposed inverse relationship between proliferation and corticosterone levels after ADX, it should be noted that the ADX effect occurs within a few days, which is clearly different from the slowly developing, opposite changes with age. Furthermore, in contrast to the effects of high corticosterone levels mediated through glucocorticoid receptor (GR) occupation, ADX involves the high affinity mineralocorticoid receptor (MR), that is occupied already by low levels of corticosterone, but that is depleted from its ligand after ADX. ADX induced apoptosis can be completely prevented by supplementing animals with low amounts of corticosterone, sufficient to occupy only MR (but not GR), or with MR-specific agonists. This indicates an important role of MR in maintaining neuronal viability. Also, ADX is associated with corticosterone absence (< 1μg/dl), whereas in intact, old animals, at least basal level as well as a normal circadian fluctuation are still present. In addition, the ADX induced increase in apoptosis could relate to the increased proliferation in the DG, as this seems to follow the peak in apoptosis. Also other studies have now shown a tight coupling between death and proliferation in the DG, e.g. following lesions, or in disorders affecting the hippocampus, like epilepsy or stroke (Gould & Tanapat, 1997; Covolan et al., 2000; Arvidsson et al., 2001). Even the induction of targeted apoptotic death induced adult neurogenesis in adult cortical neurons (Magavi et al., 2000), further confirming the close association between death and (compensatory) birth, also in areas outside the hippocampus (Kuhn et al., 2001).

Finally, as to the discrepancy between corticosterone levels and proliferation with age, other exceptions also exist, e.g. hippocampal PSA-NCAM expression is reduced with age, but does not increase after ADX (Abrous et al., 1997; Montaron et al., 1999). Therefore, although our data does not exclude that ADX of old animals may increase proliferation (Cameron & McKay, 1999), in view of the different nature of the two processes, different mechanisms probably underlie the discrepancy between proliferation changes with age and after ADX.

Adult neurogenesis represents a very low frequency phenomenon in both adult and aged rat brain (Gage, 2002; Rakic, 2002) that can be increased several-fold by ADX or enriched environmental housing (Kempermann et al., 1998b; Kempermann et al., 1998a; Cameron & McKay, 1999; van Praag et al., 1999b; van Praag et al., 1999a). Yet, several studies have proposed an important role for this phenomenon in reactivity to novelty (Lemaire et al., 1999), memory formation (Shors et al., 2001; Snyder et al., 2001) as well as clearance of memories (Feng et al., 2001), mainly in young animals. Based on our
current data, the number of newborn cells that actually reaches the DG-GCL after 4 weeks is comparable to those born in the hilus, and makes up around 0.025% of the total DG-GCL number in middle-aged animals (Fig. 3). In old animals this percentage is even less, i.e. 0.01%. When summated over a period of one month and even if the parallel cell death, subsequent division and reduced migration and differentiation are not taken into account, the numbers of new cells make up only 0.3% of the total. Further research is needed to establish whether contributions on this scale can fully explain the substantial changes in synaptic potentiation and behavior as suggested by correlative studies. These changes are likely to be due to synaptic reorganization or other modulations of synaptic transmission consistent with the proposed alternative role for neurogenesis in establishing an adaptive, long-term response for the entire hippocampal network following specific experiences, paraphrased as the addition of “strategic new gatekeepers” (Kempermann, 2002). In this respect, enriched environmental housing was recently shown to induce a 5-fold increase in the number of new neurons (Kempermann et al., 2002) even in old animals. Therefore, “deprived” housing conditions of laboratory rats are important in interpreting age-related changes found in standard housed rats. Although undoubtedly functional at the individual level (van Praag et al., 2002), further studies should be carried out to examine whether and how these very low numbers of newborn cells in adult and old animals contribute to hippocampal network function. In this respect, local environmental cues may turn out to be of crucial additional importance.

Acknowledgements

We thank Sergiu Dalm (LACDR, Leiden University, The Netherlands) for technical assistance, Dr Suresh Nair (NIBR, Amsterdam) for his corrections to the English, Wijnand Takkenberg and Dr. Erik Manders (Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences, Amsterdam) and especially Dr. Willem van Raamsdonk (Institute Neurobiology, Amsterdam) for assistance with imaging and microscopic analysis.