

Dynamics of neurogenesis in the dentate gyrus of adult rats

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Abstract

Hippocampal neurogenesis declines steadily over the first year of life in the rodent, but the process persists into senescence despite a dramatic drop in the number of neurons it produces. At this point though, the survival and development patterns exhibited by new granule cells in the aging brain remain unclear in relation to patterns observed in the younger brain. The present study was carried out in order to obtain a direct quantitative comparison of hippocampal neurogenesis in juvenile and middle-aged rats with a high degree of temporal resolution, and to compare the survival and differentiation of the new cells over time. Thirty-eight-day-old and 12-month-old, male Sprague–Dawley rats were injected with 5-bromo-2'-deoxyuridine (BrdU) in order to label cells dividing in the dentate gyrus over a 24-h period, and immunohistochemical labeling was performed in order to record cell production and survival at eight different time points over the following two-month period. Using a marker of neuronally committed precursors and immature neurons (doublecortin; DCX), as well as a marker of mature neurons (calbindin d-28 K; CaBP), the extent and timeline of neuronal differentiation, maturation, and migration of the new cells were also characterized. Results indicated that 12-month-old rats experienced a nearly 94% reduction in neurogenesis relative to juveniles, due almost entirely to a 92% drop in cell production. A largely preserved course of development and migration in the remaining newborn cells suggests treatments that enhance cell proliferation could be crucial in reversing the age-related decline in neurogenesis.

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The hippocampus is a temporal lobe brain structure involved in various types of learning and memory, and one of its sub-regions, the dentate gyrus (DG), is one of two specific brain regions to which newly generated neurons are added during adulthood [1,2]. Ongoing DG neurogenesis is a multi-stage process, encompassing a number of developmental phases including the division of precursor cells in the subgranular zone (SGZ) to produce newborn granule cells, and the subsequent differentiation and migration of the new cells within the granule cell layer (GCL). Well-characterized in many young and adult mammals including rodents (review [12]), non-human primates [10], and humans [9], neurogenesis continues throughout later life and into senescence. Interestingly though, the rate of new cell production declines steadily over time, becoming reduced at least 80% relative to young levels by about one year of age in the laboratory rat [5,14,18,22,27].

While the lifelong persistence of neurogenesis is accepted, the functional significance of the new hippocampal cells is not yet understood. It has been suggested that the cells might be involved in aspects of normal hippocampal function, such as spatial learning and memory [29]. Along these lines, decreasing neurogenesis has further been proposed as a factor in the age-related decline of cognitive ability [8]. Presently then, a specific objective in the study of hippocampal neurogenesis is the characterization of the nature and extent of the age-related changes affecting all aspects of the neurogenic process. To this point, parameters and methodologies employed in the study of neurogenesis in aging animals have been quite variable, and generalized conclusions are therefore difficult to draw.

Previous studies using aging animals have examined the survival of new neurons over time, but most [5,7,14,16,18,26,22] recorded neurogenesis at a maximum of just three widely spaced time points, or following several days of spatial learning, which in itself could affect cell counts [3,8,21].

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The present study was carried out in order to obtain a quantitative comparison of hippocampal neurogenesis in juvenile and middle-aged rats with a high degree of temporal resolution. To this end, the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) was used to label a majority of progenitor cells dividing in the DG of juvenile (38-day-old) and middle-aged (12-month-old) rats during a 24-h period (this age range covers the majority of commonly performed "adult neurogenesis" studies). New cell production and differentiation patterns of cells in the dividing populations were then assessed for each age group at eight different time points over a two-month period. Our results clarify reports from the literature and provide insight into the exact nature of age effects on the neurogenic process.

Male, Sprague–Dawley rats of two different ages were used. The "juvenile" group consisted of 24 thirty-day-old rats (96–114 g; Charles River), which were individually housed and maintained undisturbed for one week after their arrival, prior to the start of the experiment. The "middle-aged" group consisted of 24 seven-month-old rats (Harlan Sprague–Dawley), which were pair housed and maintained for 5 months after their arrival, until the start of the experiment, at which time they were 12 months of age (599–690 g). At 12 months, Sprague–Dawley rats are not yet senescent, but this group was chosen to represent the "aging" condition because the previous literature indicated that the main effects of age on neurogenesis would be evident by this time point [18,22,27]. All rats were housed in plexiglass shoebox cages with ad lib access to food and water, and were maintained on a 12:12 light:dark cycle (lights on at 7.00 a.m.).

All animals received two i.p. injections of BrdU (200 mg/kg each injection, 20 mg/ml in phosphate-buffered saline (PBS; pH 7.2) with 0.1% NaOH; Sigma), on the same day, 12 h apart (9.00 a.m. and 9.00 p.m.). Because the present study was concerned with a quantitative assessment of neurogenesis, the total BrdU dose and injection scheme were designed to maximize BrdU uptake by cells in a specific study population: those dividing in the DG within a single, 24-h period. The total BrdU dose of 400 mg/kg was higher than the conventional dose of between about 100 and 250 mg/kg, but high doses of at least 300 mg/kg of BrdU have been demonstrated as specific, quantitative, and non-toxic markers of dividing cells in the adult rat DG, while lower doses label only a fraction of the S-phase cells [6]. Further, the 400 mg/kg BrdU was split into two doses in order that a majority of the proliferating cells would be included in the labeled population, but without labeling the same cells twice. BrdU is taken up by dividing granule cells during an 8–9 h S-phase only, while the entire cell cycle lasts for about 24 h in rats [6]. Because BrdU is biologically available for about 2 h following injection, it was given in two separate, evenly spaced doses (every 12 h over a 24 h period), in order to maximize its availability for uptake by the majority of cells dividing during the 24 h period, while effectively minimizing the amount of stress experienced by the animals in response to a repeated injection procedure.

Animals were deeply anaesthetized with halothane inhalation, then transcardially perfused with 300 ml of PBS (at room temperature), followed by 200 ml of 8% paraformaldehyde at one of the following eight time points following BrdU injection: 1, 3, 7, 10, 14, 21, 28, 60 ($n=3$ for each age group at each time point). All measures were taken to minimize the possibility of pain or discomfort for the animals; animal treatments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, according to protocols approved by the University of Toronto Animal Care Committee.

Brains were removed intact immediately after perfusion, divided into two hemispheres, and post-fixed for six days in 8% paraformaldehyde. Eight percent paraformaldehyde was used in place of the more commonly used 4% in order to facilitate reliable serial sectioning with the vibratome. A pilot experiment using the same procedures but fixing one set of brains in 4% and the other in 8% paraformaldehyde demonstrated that BrdU labeling was not affected by "overfixation" with the stronger solution. The mean number of BrdU+ cells in 12-month-old animals was 671 ± 67 ($n=3$) at three days following the BrdU injection with 8% solution (see Fig. 1A), and 794 ± 137 ($n=6$) with 4% solution.

Following fixation, right hippocampal structures were removed from each brain, and coronal sections (30 μm) were cut with a vibratome along the entire dorso-ventral length of the hippocampus. Tissue sections were stored in 0.1% sodium azide in PBS at 4 °C. Sampling of sections for immunohistochemical labeling was carried out along the entire length of the DG according to a systematic random scheme. The sampling fraction was chosen to satisfy criteria described by West et al. [30], which were based on the principle that the proportion of the total variance contributed by the variability at one level of sampling (number of sections within an individual) should account for less than half the variability present at the next-higher sampling level (number of individuals within a group). According to this principle, a fraction of 1/20 (about 10 sections per hippocampus) was determined to be sufficient to produce accurate estimates in this study.

Three different immunohistochemical markers were used: BrdU, to detect the cell production and survival patterns of newly divided cells, doublecortin (DCX; a microtubule associated protein involved in differentiation and migration of young cells), to follow the development of immature granule neurons, and calbindin D-28 k (CaBP; a calcium binding protein), to indicate the maturation of granule neurons. For juvenile rats, two sets of every 20th section were double-labeled for the presence of BrdU and DCX, or BrdU and CaBP, respectively. For older rats, because BrdU-labeled cells were encountered rarely during quantification, the sampling fraction was increased to two sets of every 10th section.

All immunohistochemical labeling was performed on free-floating sections in PBS containing 0.3% Triton-X, and all incubation steps were followed with rinsing in PBS (3 \times 5 min). For BrdU labeling, sections were incubated at 45 °C for 30 min in HCL (1 M) for antigen unmasking,

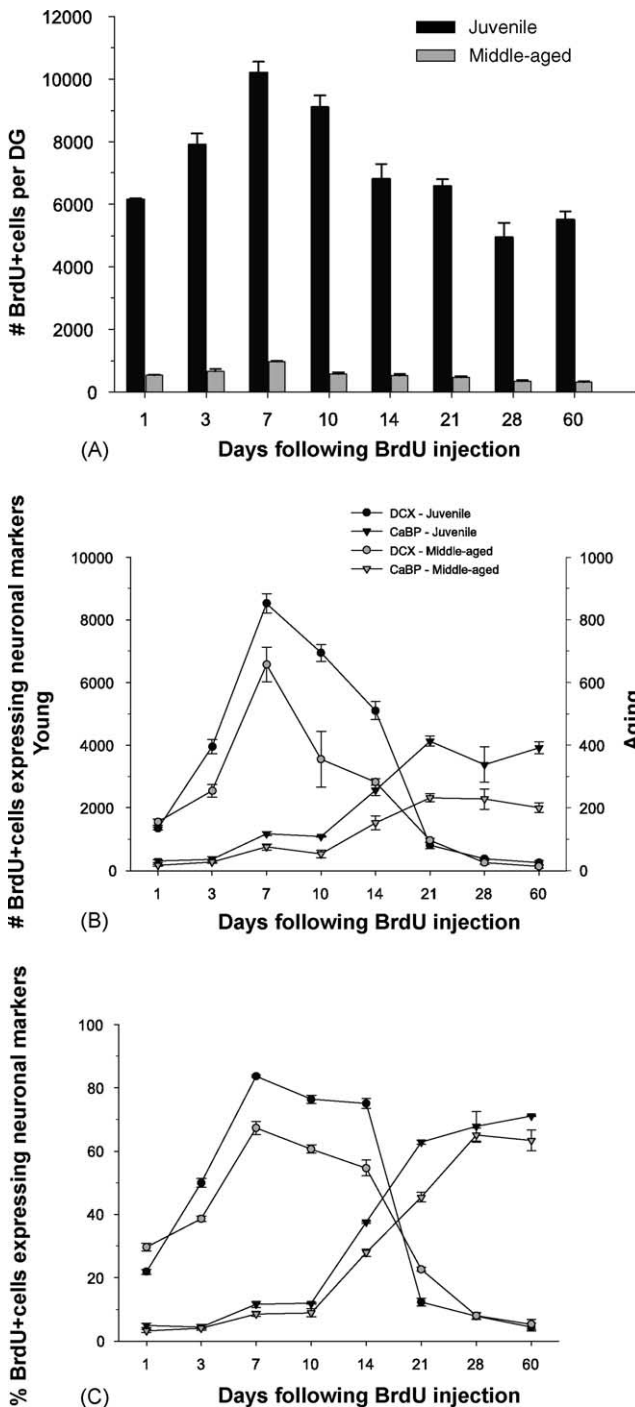


Fig. 1. Survival and development of newborn cells in the rat hippocampus. (A) Mean number of BrdU+ cells in the DG (SGZ + GCL) of juvenile and middle-aged rats over a two-month period following BrdU injection. Cell production was decreased by approximately 90% across time points in older rats relative to young, but cell survival patterns were similar. Note the peak in BrdU+ cells at 7 days after injection for both age groups, of which 54% in juvenile rats, and 33% in older rats, were still surviving at the end of the study period. (B) Mean numbers of BrdU+ cells expressing neuronal markers (DCX and CaBP) over a two-month period following BrdU injection in juvenile and middle-aged rats. Note the separate y-axes and scales for each age group. BrdU+/DCX+ cells were reduced 93% overall, and BrdU+/CaBP+ cells were reduced 93.5% overall in older rats relative to young, but temporal patterns of DCX and CaBP expression were similar. (C) Mean percentages

incubated at 4 °C for 24 h with primary antibody solution (monoclonal rat-anti-BrdU IgG, 1:200; Accurate Chemicals), then incubated at room temperature for 2 h with a secondary antibody solution (goat-anti-rat IgG, Alexa Fluor 488, 1:200, Molecular Probes; or chicken-anti-rat IgG, Alexa Fluor 594, 1:200, Molecular Probes). Sections were then rinsed for 2 min in double-distilled water (ddH₂O), and mounted onto glass slides using PermaFluor.

For double labeling with BrdU and either DCX or CaBP, sections were processed for BrdU labeling as described, then incubated at 4 °C for 24 h with primary antibody solution (polyclonal goat-anti-Doublecortin IgG, 1:200, Santa Cruz Biotechnology; or polyclonal rabbit-anti-Calbindin D-28 K IgG, 1:100, Chemicon International), then incubated at room temperature for 2 h with a secondary antibody solution (donkey-anti-goat IgG, Alexa Fluor 488, 1:200, Molecular Probes; or goat-anti-rabbit IgG, Alexa Fluor 568, 1:200, Molecular Probes). Sections were then rinsed for 2 min in ddH₂O, and mounted as described previously.

Labeled cells were counted stereologically in both the GCL and the SGZ (defined as a 20 μm-wide region bordering the GCL and the hilus) of each labeled section. In order to assess numbers of developing neurons (using BrdU- and DCX-labeled tissue), absolute numbers of BrdU+ cells and BrdU+/DCX+ double-labeled cells were counted in both juvenile and middle-aged rats. To assess cell production and numbers of mature neurons (using BrdU- and CaBP-labeled tissue), absolute numbers of BrdU+ cells and BrdU+/CaBP+ double-labeled cells were counted in both age groups. In all, the approximate number of BrdU+ cells analyzed per rat was 350 cells from 10 hippocampal sections (juvenile group), or 60 cells from 20 hippocampal sections (middle-aged group). Mean cell counts per section were multiplied by the number of sections making up the hippocampus to obtain total cell counts per DG for each animal. These counts were also used to calculate the percentage of total BrdU+ cells that expressed neuronal markers (either DCX or CaBP) at each time point.

BrdU and CaBP were visualized using a Leica DM LFS confocal microscope, while BrdU and DCX were visualized using a Nikon Optiphot 2 fluorescent microscope. In order to calculate GCL volume, GCL surface area measurements were taken for each section under the fluorescent microscope, using a Sensicam CCD camera, and Sensicontrol and ImageTool software. Total GCL volume calculation was performed for each rat using the Cavalieri method [13]. Mean GCL volumes were compared for juvenile and middle-aged rats in order to assess the possibility that a size difference in the GCL itself might contribute to differences in cell counts between the age groups.

of total BrdU+ cells expressing neuronal markers (DCX and CaBP) over a two-month period following BrdU injection in juvenile and middle-aged rats. Percent DCX expression was reduced by 15–20% at 3, 7, 10, and 14 days after BrdU injection in older rats relative to young, and percent CaBP expression was reduced by 20% at 14, 21, and 60 days after injection.

Differences between the age groups were examined using ANOVA. Two-way factorial ANOVAs (days \times group), with Tukey post hoc tests, were performed for mean BrdU+, BrdU+/DCX+, and BrdU+/CaBP+ cell counts in order to test for main effects (of time following cell birth, and of age group) on the production and development of new granule cells, and for interactions. Additionally, mean GCL volumes were compared between juvenile and middle-aged rats, using an independent sample *T*-test (SigmaStat 3.0). All graphs were prepared using Sigma Plot software.

Middle-aged rats experienced a drastic decline in neurogenesis by 12 months. Both age of rat and time following BrdU injection had significant effects on the number of BrdU-labeled cells present in the DG over a two-month period. As shown in Fig. 1A, the labeled populations produced a maximum of about 10,000 BrdU+ cells (labeled progenitors and their progeny) in the DG of juvenile rats, but a maximum of only about 1000 cells in the DG of middle-aged rats (number of BrdU+ cells at seven days after injection for juvenile rats = $10,192 \pm 373.76$; for middle-aged rats = 981 ± 8.39). Overall, the mean reduction in DG cell production across all eight time points (1–60 days following injection) was 92% in older rats relative to juvenile ($F(1,46) = 2728.146$, $P < 0.001$).

Over the 60-day study period, in terms of the relative numbers of newly generated, BrdU-labeled cells remaining at each of the time points examined, survival patterns were mainly similar between age groups. Despite a drastic drop in proliferation with age, both groups showed a significant peak in BrdU+ cells at seven days after injection ($F(7,40) = 31.181$, $P < 0.001$). Following this peak, both groups further exhibited a decrease in numbers of BrdU+ cells that by day 14 had returned to a level statistically equivalent to that seen at 1 day after injection, although this decrease was evident earlier (at day 10) in the older animals.

The large reduction in numbers of newborn granule cells between age groups was not likely to have been correlated with a change in the size of the GCL over time, since an independent samples *T*-test comparing total GCL volume indicated that the mean volume of the GCL did not differ significantly between 38-day-old and 12-month-old rats (juvenile GCL = $1.2171 \pm 0.03 \text{ mm}^3$; middle-aged GCL = $1.2779 \pm 0.05 \text{ mm}^3$; $t(46) = -1.054$, $P = 0.298$).

Immunohistochemical labeling with BrdU in combination with markers of immature neurons (DCX; [4]) or mature neurons (CaBP) allowed visualization of newly generated cells that were developing and maturing according to a neuronal fate in both juvenile and middle-aged brains. In line with the 92% age-related reduction in cell production, absolute numbers of BrdU+/DCX+ and BrdU+/CaBP+ double-labeled cells were similarly reduced in the older DG over the two-month period following cell birth. Specifically, in terms of absolute numbers, middle-aged rats experienced a 93% reduction (mean across all eight time points) in numbers of BrdU+/DCX+ cells ($F(1,46) = 2000.220$, $P < 0.001$), and a 93.5% reduction (mean across all eight time points) in num-

bers of BrdU+/CaBP+ cells ($F(1,46) = 591.036$, $P < 0.001$), relative to juveniles (Fig. 1B).

Interestingly, the temporal patterns of neuronal differentiation and maturation were largely conserved between age groups. In both groups, the absolute number of new cells expressing DCX was initially low during the days immediately after birth, before the new cells began to differentiate. BrdU+/DCX+ cell counts peaked at seven days after BrdU injection in both 38-day-old and 12-month-old rats, decreasing steadily over the next two weeks—possibly as neurons lost DCX expression during maturation (Fig. 1B). However, the drop in numbers of developing granule neurons between the 7-day and 10-day time points was steeper in middle-aged animals, probably as a consequence of the relatively increased cell death observed for aging animals during this time period. In both age groups, the absolute number of new cells expressing CaBP remained very low while the new cells were still immature during the first week following birth. BrdU+/CaBP+ cell counts increased steadily between about 10 days and 3 weeks after injection in juvenile and middle-aged rats, peaking at 21 days, and remaining steady over the remainder of the 60-day study period (Fig. 1B).

Examining neuronal differentiation overall, there was a small, age-related decline in the percentage of BrdU cells that expressed neuronal markers (~15–20% reduction in DCX expression during peak expression period at 7, 10, and 14 days after cell birth ($F(1,46) = 42.669$, $P < 0.001$; Fig. 1C); ~20% reduction in CaBP expression along developmental peak at 14 and 21 days after cell birth, and when neurons had matured at 60 days ($F(1,46) = 30.278$, $P < 0.001$; Fig. 1C).

Although neurogenesis in middle-aged rats was drastically reduced in scale relative to juvenile levels, and despite a small decrease in neuronal differentiation, the development of the remaining cells proceeded qualitatively normally. The initial increase in numbers of labeled cells between one and seven days after BrdU injection was almost certainly due to cell re-division, and may not have been seen had our high total dose of BrdU ($2 \times 200 \text{ mg/kg}$) not been used. Label dilution associated with lower doses may prevent accurate measurement of the real time course of cell proliferation.

Newborn granule cells in both juvenile and middle-aged brains appeared to progress through similar series of overlapping developmental stages. A majority of new cells in both age groups expressed DCX within one week after cell birth (percentage of BrdU+ cells co-labeled for DCX at 7 days after injection in juvenile rats = 84%; in middle-aged = 67%). Additionally, most new cells expressed CaBP within one month (percentage of BrdU+ cells co-labeled for CaBP at 28 days in juvenile rats = 68%; in middle-aged = 65%). For both age groups, the first month (28 days) following cell birth was marked by the elimination of up to half the newborn cells (reduction in BrdU+ cells between 7 days (peak), and 28 days after injection for juvenile rats = ~50%; for middle-aged = ~35%; $F(1,46) = 2728.146$, $P < 0.001$), and increasing expression of a mature neuronal marker in the remaining cells.

The qualitative development of new granule cells during the two-month period following cell birth is illustrated in Fig. 2 in supplementary materials on line, using examples from the younger animals. Again, dramatically fewer but similar examples were recorded at all time points in the middle-aged group (not shown). Furthermore, the timeline and pattern of new cell migration were similar in juvenile and middle-aged rats. Specifically, the tendency of developing granule cells in the DG of 38-day-old rats to migrate radially from their birthplace in the SGZ, reaching their destination within the GCL by three to four weeks after birth, appeared to be conserved in 12-month-old rats.

Our results support a dramatic, 94% decline in net neurogenesis (92% decrease in cell production) over the first year of life in the rat, with a nearly completely preserved course of development and migration in the remaining newborn cells. Based on the expression of mature neuronal marker CaBP, dividing progenitor cells in the hippocampus of a juvenile rat (38 days old) are capable of producing approximately 4000 new neurons per day. These neurons are likely to persist in the DG for at least several months, based on their survival for at least two months in the present study, and supported by previous reports indicating that adult-generated granule neurons surviving to maturity are very stable, and may permanently replace more mature granule cells [7]. By middle age (12 months old), declining neurogenesis reduces production to only about 250 new neurons each day.

In this study, the different developmental stages of the process were not equally affected by aging. Most significantly influenced was granule cell production, which became decreased by 92% over a 12-month period, in agreement with previous findings [18,27,31]. Because cell production is so profoundly reduced with aging, the mechanisms of the age-related decrease in dentate neurogenesis are likely to involve changes in the progenitor cell population over time. But the nature of these changes is unclear. One hypothesis assumes that the neural precursor cells located in the DG are not true stem cells but restricted progenitors with a limited potential for self-renewal. Consequently, the population from which new cells can be produced may become progressively reduced over time, leading to the decrease in neurogenesis with increasing age [25]. By contrast in the SVZ, populated in part by indefinitely self-renewing stem cells, the age-related reduction in neurogenesis (~50%) is reported to be much less pronounced than it is in the hippocampus [20]. But work showing that the age-related decrease in granule cell production can be reversed by adrenalectomy [5] or by seizure induction [11] suggests that progressive lengthening of the cell cycle time in intact progenitors, perhaps in response to tonic inhibition by rising levels of corticosteroids, could also account for the decline. Alternatively, an increasing proportion of progenitors might become quiescent with age, but retain their potential for reactivation.

Uniquely, our study was designed to examine the age impact on further stages of the neurogenic process, compar-

ing complete patterns of neuronal development between age groups with high temporal resolution. A notable difference observed between groups was that the decline in cell production was marked by increased cell death between 7 and 10 days after cell birth in middle-aged rats relative to juveniles. This suggests that the pool of immature cells from which new neurons can be recruited may become more quickly depleted with aging, especially just before the important period of transition during which immature neurons begin to express mature neuronal markers. This phase in the development of new granule cells is significant, because cells that survive long enough to express mature neuronal markers are reported to persist for at least several months in the DG [2,7,16]. Indeed, relative to younger animals, the middle-aged rats in this study also displayed a 20% drop in the proportional expression of a mature neuronal marker.

In spite of some important methodological differences, our main finding of a preserved ability of adult-born neurons to differentiate, mature, and migrate into the GCL in aging brains largely agrees with the recent study of Rao et al. [24]. Our 24 h BrdU injection protocol allows for higher temporal resolution of the early stages of neurogenesis during the first 2–3 weeks after cell birth, while the 12-day BrdU protocol utilized by Rao et al. may allow more extensive sampling of newborn cells, perhaps giving a more reliable estimate of the neurons' long term survival (in months). In spite of differences in protocol, both studies revealed that the main difference between the juvenile/young and middle-aged rats concerns the rates of cell production, while differentiation and maturation rates remain similar. A small delay in maturation during the third week after cell birth was also seen in both studies. By contrast, Heine et al. [14] reported a drastic decline in neuronal maturation, in addition to reduced proliferation, in middle-aged rats. One possible reason for this discrepancy may be the use of Wistar rats in the Heine et al. study, while Sprague–Dawley and Fisher strains were used here and by Rao et al. [24].

Overall, our results provide quantitative information about the nature of the age-related decrease in hippocampal neurogenesis by direct comparison, and suggest that the temporal patterns of neuronal development and migration remain mostly unchanged between 38 days and 12 months in Sprague–Dawley rats, except for a 20% decrease in differentiation. This small drop in proportional expression of DCX and CaBP could be partly due to the reduced proportion of progenitors destined to become neurons: alternative pathways of development include the formation of glial or endothelial cells [17,23,28]. Regardless, since adult-born granule cells appear capable of migrating and developing along a normal course, even into old age, manipulations that enhance cell proliferation may be sufficient to restore the declining neurogenesis levels in the aging hippocampus. Indeed, adrenalectomy [5], blockade of NMDA receptors [22], insulin-like growth factor-1 [19], and neurotrophins FGF-2 and HB-EGF [15] have all been shown to increase neurogenesis in aging animals primarily by boosting cell proliferation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neulet.2005.05.022](https://doi.org/10.1016/j.neulet.2005.05.022).

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