Published in final edited form as:

Neurobiol Aging. 2008 January ; 29(1): 129–147. doi:10.1016/j.neurobiolaging.2006.09.015.

Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus

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Abstract

To investigate whether dramatically waned dentate neurogenesis during aging is linked to diminution in neural stem/progenitor cell (NSC) number, we counted cells immunopositive for Sox-2 (a putative marker of NSCs) in the subgranular zone (SGZ) of young, middle-aged and aged F344 rats. The young SGZ comprised ~50,000 Sox-2⁺ cells and this amount did not diminish with aging. Quantity of GFAP⁺ cells and vimentin⁺ radial glia also remained stable during aging in this region. Besides, in all age groups, analogous fractions of Sox-2⁺ cells expressed GFAP (astrocytes/NSCs), NG-2 (oligodendrocyte-progenitors/NSCs), vimentin (radial glia), S-100 β (astrocytes) and doublecortin (new neurons). Nevertheless, analyses of Sox-2⁺ cells with proliferative markers insinuated an increased quiescence of NSCs with aging. Moreover, the volume of rat-endothelial-cell-antigen-1⁺ capillaries (vascular-niches) within the SGZ exhibited an age-related decline, resulting in an increased expanse between NSCs and capillaries. Thus, decreased dentate neurogenesis during aging is not attributable to altered number or phenotype of NSCs. Instead, it appears to be an outcome of increased quiescence of NSCs due to changes in NSC milieu.

Keywords

Keywords: Astrocytes; 5'-Bromodeoxyuridine; Dentate neurogenesis; Doublecortin; GFAP; Hippocampus; Ki67; Neural progenitors; NG-2; Oligodendrocytic progenitors neural stem cells; RECA-1; Sox-2; Subgranular zone; Vimentin

1. Introduction

Addition of fresh granule cells to the dentate gyrus (DG) of the hippocampus takes place throughout life [1,50]. However, the rate of hippocampal neurogenesis wanes radically by middle age [52,62,68], which may play a role in impairments of hippocampal-dependent learning and memory function observed during old age [3,17,86; however, see 4,5,58]. Dentate neurogenesis encompasses proliferation of stem/progenitor cells (NSCs) in the

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Disclosure statement: (a) We do not have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence (bias) their work. (b) All animal experiments in this study were performed as per the animal protocol approved by the Institutional Animal Care and Use Committees of the Duke University Medical Center and the Durham Veterans Affairs Medical Center.

subgranular zone (SGZ), survival and neuronal differentiation of newly born cells, migration of newly differentiated neurons into the granule cell layer (GCL), and functional maturation of newly introduced neurons in the GCL [8,10,21,30,46,85]. Substantial changes in some or all of the above events may underlie the decreased neurogenesis discerned in aging. A recent study in rats suggests that age-related decrease in dentate neurogenesis is primarily attributable to decreased production of new cells, as the extent of neuronal differentiation from newly born cells, and the migration and long-term survival of newly born neurons are analogous between young, middle-aged and aged groups [68]. Nevertheless, the precise reasons for striking decreases in the production of new cells from NSCs at middle age are unknown. This might be due to multiple changes that occur in the hippocampus at middle age. These include decreased concentration of distinct neurotrophic factors and signaling proteins that are known to promote proliferation of NSCs [37,76] and an increased concentration of glucocorticoids [9; but see 7]. Additionally, it is plausible that a decrease in the number of NSCs during the course of aging contributes to age-related decrease in DG neurogenesis. However, no studies on changes in the number of NSCs in the SGZ during middle age and old age are available, apparently due to lack of apt markers for identifying NSCs in vivo.

Recently, the transcription factor Sox-2 has been proposed as a marker of NSCs [6,49]. Sox-2 is a member of the Sox (sex determining region of Y-chromosome) gene family, which encode transcription factors regulating crucial developmental decisions in different systems [44]. Sox-2 is expressed in the totipotent stem cells of the inner cell mass and other early multipotent cell lineages [2,19,66,89]. Sox-2 also serves as a marker of the developing neural tube [2,48] and embryonic stem cells [26]. Furthermore, a recent study shows that Sox-2 positive (Sox-2⁺) cells isolated from the developing and adult brain readily form neurospheres in vitro, which can be passaged for extended periods or pushed to differentiate into neurons, astrocytes and oligodendrocytes by changing culture conditions [6]. Although examination of the adult brain in vivo reveals Sox-2 expression in both neural progenitors and a fraction of astrocytes, Sox-2 is considered a good marker for identifying NSCs in neurogenic regions, particularly when examined in combination with markers of astrocytes and glial progenitors [6,49].

In this study, to ascertain the age-related changes in the quantity of NSCs, we employed Sox-2 immunostaining and quantified the number of Sox-2⁺ cells in the SGZ of young adult (4-months old), middle-aged (12-months old) and aged (24-months old) F344 rats using the optical fractionator cell counting method. In addition, to determine the age-related changes in the phenotype of Sox-2⁺ cells of the SGZ, we quantified the percentages of Sox-2⁺ cells expressing GFAP (astrocytes and NSCs), vimentin (radial glia), NG-2 (oligodendrocytic progenitors and NSCs), S-100 β (mature astrocytes), rip (mature oligodendrocytes), and doublecortin (newly born neurons) within the SGZ. As additional measures of NSC number, we quantified the numbers of GFAP⁺ cells (presumably stem/progenitor cells and local astrocytes) located in the SGZ and vimentin⁺ radial glia in the SGZ and granule cell layer (GCL) of the DG. This is appropriate considering the idea proposed in earlier studies that a fraction of GFAP⁺ cells and radial glia exhibit characteristics of NSCs [14,16,56,57], and vimentin is a marker of radial glia [36,82]. Furthermore, we discerned the proliferative status of NSCs in different age groups by analyzing the fractions of Sox-2⁺ cells expressing Ki67 (an endogenous marker of proliferation) and 5'-bromodeoxyuridine (BrdU; an exogenous marker of proliferation). Additionally, as vascular niches in the SGZ are considered to be important for neurogenesis and capillaries are one of the major components of vascular niches [18,40,65], we quantified age-related changes in the volume of rat endothelial cell antigen-1 immunopositive (RECA-1⁺) capillaries within the SGZ and also measured percentages of Sox-2⁺ cells that are located adjacent to RECA-1⁺ capillaries in the SGZ of young, middle-aged and aged animals.

2. Methods

2.1. Animals and collection of brain tissues

Male Fischer 344 rats were obtained from the National Institute of Aging colony at Harlan Sprague–Dawley (Indianapolis, IN). Three groups of rats were used in this study: young adult (4-months old; $n = 5$), middle-aged (12-months old; $n = 5$), and aged (24-months old; $n = 5$). F344 rats were chosen in this study because the genetic background of this strain is known, and the normal life span and development of these rats are reasonably well defined [13]. The experiments were performed as per the animal protocol approved by the Institutional Animal Care and Use Committees of the Duke University Medical Center and the Durham Veterans Affairs Medical Center. For collection of brain tissues, rats were deeply anesthetized with halothane and perfused through the heart with 4% paraformaldehyde solution. The brains were post-fixed in 4% paraformaldehyde overnight and were cryoprotected using 30% sucrose solution. Thirty-micrometer-thick cryostat sections were cut coronally through the entire hippocampus and collected serially in phosphate buffer (PB).

2.2. Sox-2, GFAP, vimentin and RECA-1 immunohistochemistry

Four separate sets of serial sections (every 20th) through the entire hippocampus of young, middle-aged and aged F344 rats ($n = 5/\text{group}$) were chosen and processed for Sox-2, vimentin, GFAP and RECA-1 immunostaining. Free-floating sections were treated first with phosphate buffered saline (PBS) containing 20% methanol and 3% hydrogen peroxide for 30 min and rinsed thoroughly in PBS. Sections were then incubated in 10% normal serum in PBS containing 0.1% Triton X-100 for 30 min and incubated overnight at 4 °C in the respective primary antibody solution, prepared using the following dilutions: rabbit anti-sox-2, 1:500 (Chemicon, Temecula, CA), mouse anti-vimentin, 1:1000 (Chemicon), rabbit anti-GFAP, 1:1000 (Dako, Carpinteria, CA) and mouse anti-RECA-1, 1:200 (Serotec, Raleigh, NC). The antibody used for Sox-2 staining in this study was used previously for characterizing NSCs in the SGZ of the adult hippocampus [49]. As per the manufacturers data sheet, it is an affinity purified IgG raised in rabbit and recognizes a 34 kDa band corresponding to Sox-2 that is not observed in cytosolic extract. The antibody used for RECA-1 immunoreactivity in this study was successfully used for staining brain capillaries in previous studies [22,61]. Following incubation in the primary antibody, sections were rinsed three times in PBS, incubated for an hour in the respective secondary antibody solutions (biotinylated anti mouse IgG [Vector Labs, Burlingame, CA] for vimentin and RECA-1, and biotinylated anti-rabbit IgG [Vector Labs] for Sox-2 and GFAP), washed thrice, and treated with the avidin–biotin complex (ABC) reagent for an hour. The immunohistochemical reaction was visualized using vector gray (Vector Labs) as chromogen and the sections were mounted on slides and air-dried. To clearly demarcate the SGZ from the GCL and also to visualize the soma of GFAP⁺ astrocytes, immunostained sections were counterstained using nuclear fast red, dehydrated, cleared and coverslipped using permount. To exclude any possible effects of the staining protocol on visualization of the immunoreactive structures, sections from the three age groups were processed in parallel with identical concentrations of primary and secondary antibody solutions and the ABC reagent. Furthermore, the concentrations of vector gray and hydrogen peroxide for the chromogen reaction were kept constant for all groups. Negative control sections for each antigen were processed in the same manner except that the primary antibody incubation step was replaced by continued incubation in the normal serum. Neither immunostaining nor any recognizable background staining was observed under these conditions in negative control sections.

2.3. Quantification of Sox-2⁺, vimentin⁺ and GFAP⁺ cells using the optical fractionator method

We quantified numbers of Sox-2⁺ and GFAP⁺ cells in the entire SGZ, and the number of vimentin⁺ primary radial glial processes reaching the molecular layer through the GCL in each rat belonging to the three age groups ($n = 5/\text{age group}$). Every 20th section through the entire hippocampus was measured via the optical fractionator counting method using the StereoInvestigator System (Microbrightfield, Williston, VT). A section at the commencement of the hippocampus (anterior most part of the hippocampus) served as the first section in all animals chosen for quantification. Because we employed serial sections (every 20th) through the entire septotemporal axis of the hippocampus for these studies, our Sox-2⁺ and GFAP⁺ cell counts reflect the total number of labeled cells for the entire SGZ, whereas our counts of vimentin⁺ primary radial glial processes provide an indirect measure of vimentin⁺ radial glia in the SGZ and GCL.

The StereoInvestigator System consisted of a color digital video camera (Optronics, Muskogee, OK) interfaced with Nikon E600 microscope. In every animal, Sox-2⁺ and GFAP⁺ cells and vimentin⁺ primary radial glial processes within the demarcated regions were counted from 10 to 100 frames (each measuring $20\ \mu\text{m} \times 20\ \mu\text{m}$ for Sox-2 and GFAP, and $40\ \mu\text{m} \times 40\ \mu\text{m}$ for vimentin) in each of the selected sections using the 100 $\times$ oil immersion objective lens. The frames were selected using the systematic random sampling scheme, which provides an unbiased and efficient sampling technique [37]. The number and density of frames were selected using the optical fractionator component of the Stereo Investigator system. The original thickness of cryostat sections used in this study was 30 μm . However, the average thickness of sections was reduced to 53% of the initial section thickness following immunostaining, dehydration and clearing in all age groups. Hence, at the time of data collection, the thickness of sections was decreased from 30 to 16 μm . For counting Sox-2⁺ and GFAP⁺ cells in the SGZ, the contour of this region (~two-cell thick zone [40 μm zone] between the junction of the GCL and the dentate hilus) was first delineated using the tracing tool of the StereoInvestigator. The inner rim of the GCL formed the outer margin of this zone, whereas a parallel line drawn at 40 μm distance (~two-cell depth) from the inner rim of the GCL served as the internal margin of this zone. For counting vimentin⁺ primary radial glial processes reaching the molecular layer through the GCL, both SGZ and GCL were demarcated. The optical fractionator component was then activated, and the number and location of counting frames and the counting depth were determined by entering parameters such as the grid size (60 $\mu\text{m} \times 60\ \mu\text{m}$ for Sox-2 and vimentin; 150 $\mu\text{m} \times 150\ \mu\text{m}$ for GFAP), the thickness of guard zone (4 μm) and the optical dissector height (8 μm). A computer-driven motorized stage then allowed the section to be analyzed at each of the counting frame locations. In every counting frame location, the top of the section was set, after which the plane of the focus was moved 4 μm deeper through the section (guard zone) to prevent counting inaccuracies due to uneven section surface. This plane served as the first point of the counting process. All Sox-2⁺ and GFAP⁺ cells that came into focus in the next 8 μm section thickness were counted if they were entirely within the counting frame or touching the upper or right side of the counting frame. Identification of Sox-2⁺ cells during counting is straightforward because of clear immunostaining in the nucleus of these cells whereas in GFAP⁺ cells, cell body or nuclear staining is not discernable though the position of soma is typically indicated by a junction where four to five thick GFAP⁺ processes emerge. In this study, we employed neutral red counterstaining following GFAP immunostaining, which facilitated identification of the position of soma by the presence of neutral red positive nucleus at or adjoining the junction where four to five thick GFAP⁺ processes emerge. For vimentin⁺ primary radial glial processes, we counted only primary processes that are predominantly inside the dissector (i.e. within the $40\ \mu\text{m} \times 40\ \mu\text{m} \times 8\ \mu\text{m}$ volume). This modification in the counting method

was necessary, as the soma of radial glial cells were mostly indistinct with vimentin immunostaining. As the length of vimentin⁺ primary radial glial processes ranged from 20 to 30 μm in all age groups, the above protocol was efficient for making an unbiased estimate of the number of radial glia in the SGZ and GCL.

Based on the above parameters and counts, the StereoInvestigator program calculated the total number of Sox-2⁺ and GFAP⁺ cells and vimentin⁺ primary radial glial processes per selected region, using the optical fractionator formula, $N = 1/ssf \cdot 1/asf \cdot 1/hsf \cdot EQ^-$ [37,68]. The abbreviation ssf represents the section sampling fraction, which is 20 in this study; asf symbolizes the area sampling fraction, which is calculated by dividing the area sampled with the total area of the layer; hsf stands for the height sampling fraction, which is calculated by dividing the height sampled (i.e. 8 μm in this study) with the section thickness at the time of analysis (i.e. 16 μm); EQ⁻ denotes the total count of particles sampled for each region. An option in the Stereo Investigator program allowed the experimenter to remain unaware of the running cell count totals until all sections for each animal were completed. In this study, average EQ⁻ was 139 for Sox-2⁺ cell counting, 77 for GFAP⁺ cell counting and 65 for counting of vimentin⁺ primary radial glial processes. Additionally, both Gundersen coefficient of error (CE) and Schmidt-Hof CE ranged from 0.05 to 0.1 in all age groups. The data between young adult ($n = 5$), middle-aged ($n = 5$), and aged ($n = 5$) animals were compared using one-way analysis of variance (ANOVA) with Student–Newman–Keuls multiple comparisons post hoc test. All data are presented as means $\pm$ standard errors (S.E.M.).

2.4. Analyses of Sox-2⁺ cells expressing GFAP, S-100 β , NG2, vimentin, rip and doublecortin

We analyzed whether Sox-2⁺ cells in the SGZ display other proteins such as GFAP (marker of astrocytes and NSCs), S-100 β (marker of mature astrocytes), NG2 (marker of oligodendrocytic progenitors and NSCs), vimentin (marker of radial glia), rip (marker of mature oligodendrocytes), and doublecortin (marker of newly born neurons) using dual immunofluorescence and confocal microscopy. For analyses of each of the antigens (GFAP, S-100 β , NG2, vimentin, rip, or doublecortin), four representative sections from each animal ($n = 4/\text{age group}$) were processed and analyzed. All sections were first incubated overnight in the Sox-2 primary antibody solution (rabbit anti-Sox-2; Chemicon), washed in PBS, treated with biotinylated anti-rabbit IgG (Vector Labs) for 1 h, rinsed thrice in PBS and incubated for 1 h in streptavidin fluorescein solution (Molecular Probes, Carlsbad, CA) for sections chosen for analyses of Sox-2⁺ cells expressing GFAP, S-100 β , NG-2, vimentin and rip. However, for sections chosen for analyses of Sox-2⁺ cells expressing doublecortin, streptavidin Texas red solution (Molecular Probes) was used.

Following visualization of Sox-2 immunofluorescence, sections were washed thoroughly in PBS and processed for second immunofluorescence for visualization of antigens such as GFAP, S-100 β , NG-2, vimentin, rip and doublecortin. The primary antibodies comprised mouse mono-clonals against GFAP, S-100 β , NG-2 and vimentin purchased from Chemicon, a mouse monoclonal against rip purchased from Hybridoma Bank (Iowa City, IA), and a goat polyclonal against doublecortin purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sections were incubated for 48 h in the primary antibody, washed in PBS, treated with the appropriate secondary antibody (goat anti-mouse Alex flour 594 [Molecular Probes] for GFAP, S-100 β , NG-2, vimentin and rip, and rabbit anti-goat Alexa flour 488 [Molecular Probes] for doublecortin) for 1 h, rinsed thoroughly in PBS and mounted on glass slides using slowfadeantifade mounting medium (Molecular Probes). The sections were examined and analyzed, using a laser confocal microscope (LSM-410 Carl Zeiss). The fractions of Sox-2⁺ cells expressing GFAP, vimentin, NG-2, S-100 β , rip and doublecortin were measured separately. For determination of co-expression of Sox-2 with other antigens,

Sox-2⁺ cells in the SGZ were individually examined (100 cells/animal for every antigen, 4 animals/age group) using Z-sectioning at 1 μ m intervals. The optical stacks of 8–10 images were used for determination of dual antigen labeling.

2.5. Analyses of the proliferative status of Sox-2⁺ cells via Sox-2 and Ki67 dual immunofluorescence

We discerned the proliferative status of NSCs in different age groups by analyzing the fractions of Sox-2⁺ cells expressing Ki67, an endogenous marker of cell proliferation. Representative sections from all animals (4 sections/animal; 4 animals/age group) were blocked for 30 min in PBS solution containing 5% normal horse serum and 5% normal goat serum. Following this, sections were incubated overnight in a cocktail of primary antibodies against Ki67 (rabbit monoclonal from Vector Labs; 1:200) and Sox-2 (goat polyclonal from Santa Cruz Biotechnology; 1:1000) at 4 °C, rinsed thoroughly in PBS and treated with a mixture of anti-rabbit Alexa flour 594 (Molecular Probes; 1:100) and biotinylated anti-goat IgG (Vector Labs; 1:250) for 60 min. Sections were then washed in PBS, incubated in streptavidin fluorescein solution (Molecular Probes; 1:200) for 60 min, rinsed again in PBS, and mounted on clean slides using slowfade-antifade mounting medium (Molecular Probes). Using a laser confocal microscope (LSM-410 Carl Zeiss), the sections were examined and fractions of Sox-2⁺ expressing Ki67 were measured. For this, the presence of Ki67 in Sox-2⁺ cells of the SGZ were individually examined (100 cells/animal; 4 animals/age group) using Z-sectioning at 1 μ m intervals. The optical stacks of five to eight images were used for determination of dual antigen labeling.

2.6. Analyses of the proliferative status of Sox-2⁺ cells via BrdU labeling methods

Young adult, middle-aged, and aged male F344 rats ($n = 4$ /age group) received four intraperitoneal injections of BrdU (1 injection every 6th hour for 18 h at a dose of 100 mg/kg b.w.). At 6 h after the last BrdU injection, rats were perfused with 4% paraformaldehyde solution and 30 μ m thick serial sections were cut coronally through the hippocampus using a cryocut. Representative sections from all animals (4 sections/animal) were processed for BrdU and Sox-2 dual immunofluorescence. Sections were first incubated in formamide (50%) solution prepared in 2 $\times$ saline sodium citrate buffer for 2 h at 65 °C, washed in Tris-buffered saline (TBS), and incubated in 2N HCl for 60 min at 37 °C. The sections were then neutralized with borate buffer (0.1 M, pH 8.5), washed in PBS, blocked in 10% normal goat serum, incubated overnight at 4 °C in a cocktail of primary antibodies against BrdU (rat anti-BrdU from Serotec; 1:200) and Sox-2 (rabbit anti-Sox-2 from Chemicon 1:500). The sections were washed in PBS, incubated in a mixture of secondary antibodies (anti-rat Alexa flour 594 [Molecular Probes; 1:200]) and biotinylated anti-rabbit IgG [Vector Labs]) for 1 h, washed in PBS, and treated with streptavidin fluorescein solution (Molecular Probes; 1:200) for 1 h. Sections were washed thoroughly in PBS and mounted on clean slides using slowfadeantifade mounting medium (Molecular Probes). The sections were examined using a laser confocal microscope (LSM-410 Carl Zeiss), and fractions of Sox-2⁺ cells expressing BrdU were quantified via examination of the presence of BrdU in individual Sox-2⁺ cells (100 cells/animal; 4 animals/age group) of the SGZ using Z-sectioning at 1 μ m intervals. The optical stacks of five to eight images were used for determination of Sox-2 and BrdU dual labeling.

2.7. Measurement of the volume fraction of RECA-1⁺ capillaries within the SGZ using Neurolucida

To elucidate age-related changes in the amount of the major component of vascular niches within the SGZ, we measured the volume fraction of the SGZ occupied by RECA-1⁺ capillaries in serial sections (every 20th) of the hippocampus from all three age groups of rats ($n = 5$ /age group). In every section, the contour of the SGZ (two-cell thick zone [~ 40

μm] between the junction of GCL and dentate hilus) was first delineated using the tracing tool of the NeuroLucida program. Following this, every RECA-1⁺ capillary within the demarcated SGZ was traced individually using a different color. Six serial sections (every 20th) were separately traced and saved in each animal belonging to the three groups ($n = 5$ /age group). Using NeuroExplorer component of NeuroLucida program, the contour summary (i.e. measurements of SGZ volume and total volume of RECA-1⁺ capillaries) was obtained for each section, and the volume fraction of RECA-1⁺ capillaries occupying the SGZ was calculated for every animal in each group. The data were compared between the three age groups using one-way ANOVA.

2.8. Measurement of the relationship between Sox-2⁺ cells and RECA-1⁺ capillaries within the SGZ

To ascertain age-related changes in the relationship between Sox-2⁺ cells and RECA-1⁺ capillaries within the SGZ, we measured percentages of Sox-2⁺ cells that are located adjacent to RECA-1⁺ capillaries in the SGZ of young, middle-aged and aged animals. Representative sections from all animals (4 sections/animal; 4 animals/age group) were first processed for Sox-2 and RECA-1 dual immunofluorescence. For this, the sections were blocked in 10% normal goat serum for 30 min, incubated overnight at 4 °C in a cocktail of primary antibodies to Sox-2 (rabbit polyclonal from Chemicon; 1:200) and RECA-1 (mouse monoclonal from Serotec; 1:200). Sections were then washed in PBS, treated with a mixture of secondary antibodies (anti-mouse Alexa flour 594 [Molecular Probes; 1:100] and biotinylated anti-rabbit IgG [Vector Labs; 1:250]) for 1 h, rinsed in PBS, incubated in streptavidin fluorescein solution (Molecular Probes; 1:200) for 1 h and mounted using slowfade-antifade mounting medium. Sections were then examined in a laser confocal microscope (LSM-410, Carl Zeiss) and percentages of Sox-2⁺ cells that are located adjacent to RECA-1⁺ capillaries were measured via examination of the location of individual Sox-2⁺ cells (100 cells/animal; 4 animals/age group) in relation to RECA-1⁺ capillaries within the SGZ using Z-sectioning at 1 μm intervals.

3. Results

3.1. Distribution and number of Sox-2⁺ cells in the SGZ of the DG

Immunohistochemical analysis revealed Sox-2⁺ nuclei in all layers of the young adult DG, which is consistent with previous reports [6,49]. However, the density of Sox-2⁺ cells was conspicuously greater in the SGZ, in comparison to the dentate hilus and the dentate molecular layer (Fig. 1(A1, B1, C1)). The distribution of Sox-2⁺ cells was scarce in the dentate granule cell layer (Fig. 1(A1, B1, C1)). In the middle-aged and aged DG, both distribution and density of Sox-2⁺ cells appeared comparable to that in the young adult DG (Fig. 1(A1–C2)). In addition to the DG, some Sox-2⁺ cells were also found scattered in different layers of the CA1 and CA3 subfields of the hippocampus of all age groups of animals (data not illustrated), which is consistent with the expression of Sox-2 in a small fraction of hippocampal astrocytes demonstrated for young animals in a previous study [49]. As Sox-2 has been proposed as a marker of NSCs in the neurogenic SGZ of the DG [6,49], we quantified the absolute number of Sox-2⁺ cells in the SGZ of animals belonging to all three age groups, using serial sections through the entire hippocampus and the optical fractionator method. The SGZ of young adult (4-months old) animals contained an average of 51,120 Sox-2⁺ cells (mean ± S.E.M. = 51,120 ± 1984, $n = 5$; Fig. 1(D)). Interestingly, comparable number of Sox-2⁺ cells were also found in the SGZ of middle-aged (12-months old, 51,480 ± 1988, $n = 5$) and aged (24-months old, 47,520 ± 1948, $n = 5$) animals (Fig. 1(D)). Thus, the number of Sox-2⁺ cells in the SGZ remains stable between young adult age and middle age as well as between middle age and old age.

3.2. Phenotypical features of Sox-2⁺ cells in the DG

To determine whether aging alters the phenotypical features of Sox-2⁺ cells in the DG, we performed dual immunofluorescence assays for Sox-2 and several neural antigens such as GFAP (marker of astrocytes and NSCs), S-100 β (marker of mature astrocytes), NG2 (marker of oligodendrocytic progenitors and NSCs), vimentin (marker of radial glia), rip (marker of mature oligodendrocytes) and doublecortin (marker of newly born neurons). Fig. 2 illustrates examples of Sox-2⁺ cells in the young and aged SGZ expressing GFAP (A1–J3), S-100 β (K1–L3), and NG2 (M1–N3). Analyses using Z-sectioning in a confocal microscopy revealed that only fractions of Sox-2⁺ cells express these antigens in all age groups, as Sox-2⁺ cells exhibiting GFAP/S-100 β /NG2 could be seen alongside Sox-2⁺ cells lacking GFAP/S-100 β /NG2 expression (Fig. 2). Some of the Sox-2⁺ cells that expressed GFAP appeared to have radial glial morphology (Fig. 2(A2–C3)), whereas virtually all Sox-2⁺ cells expressing S-100 β resembled mature astrocytes (Fig. 2(K2–L3)). A vast majority of Sox-2⁺ cells expressing NG2 appeared to have the morphology of oligodendrocyte progenitors (Fig. 2(M2–N3)). Thus, the presence of S-100 β /NG2 in fractions of Sox-2⁺ cells suggests that, in addition to detecting NSCs, Sox-2 immunostaining labels fractions of mature astrocytes and oligodendrocytic progenitors.

The Sox-2⁺ cells expressing vimentin in the SGZ and GCL displayed radial glial morphology in all age groups (Fig. 3(A1–B3)). Interestingly, Sox-2 immunoreactivity was present in all vimentin⁺ cells examined in the SGZ and GCL. Examination of rip⁺ cells in sections processed for Sox-2 and rip dual immunofluorescence revealed that none of the Sox-2⁺ cells in the SGZ expressed rip (Fig. 3(C1–C3)). Thus, mature oligodendrocytes in the SGZ do not exhibit Sox-2 expression. Furthermore, a vast majority of Sox-2⁺ cells lacked doublecortin expression. However, careful analyses of doublecortin⁺ cells using Z-sectioning revealed Sox-2 expression in ~2% of newly born neurons in all age groups. In doublecortin⁺ neurons expressing Sox-2, the Sox-2 expression was relatively less intense in the nucleus and doublecortin⁺ dendrites either were absent or aligned horizontally along the inner margin of the granule cell layer (Fig. 3(D1–D3)). As these dendritic features represent an immature phenotype of newly born granule cells [67–69], it appears that only very immature newly born granule cells (i.e. during the early phase of doublecortin expression) express Sox-2. This possibility is supported by the observation that doublecortin immunoreactive neurons exhibiting vertical dendrites extending into the dentate molecular layer (presumably relatively mature newly born neurons) do not exhibit Sox-2 immunoreactivity in their nuclei (Fig. 3(E1–F3)). Thus, relatively mature and post-mitotic newly born neurons in the SGZ do not express Sox-2.

We quantified the fractions of Sox-2⁺ cells expressing GFAP, vimentin, NG2, and S-100 β in all age groups using confocal microscopy and Z-section analyses of Sox-2⁺ cells (~100 cells/animal, 4 animals/age group) in the SGZ. The results suggest that 51–52% of Sox-2⁺ cells exhibit GFAP, 8–9% display vimentin, 28–34% exhibit S-100 β and 32–35% display NG2 in all three age groups of animals (Fig. 4). Thus, fractions of Sox-2⁺ cells expressing GFAP, vimentin, NG2, or S-100 β remain stable during the course of aging, suggesting that aging does not alter the phenotypical features of Sox-2⁺ cells in the SGZ.

3.3. Proliferative status of Sox-2⁺ cells in the SGZ

We visualized Sox-2⁺ cells expressing the proliferative marker Ki67 in the subgranular zone using Sox-2 and Ki67 dual immunofluorescence and confocal microscopy (Fig. 5(A1–B3)). The Ki67 cells in the SGZ appeared in clusters and all Ki67⁺ cells were immunopositive for Sox-2. In the young SGZ, the clusters were more frequent and individual clusters invariably comprised greater numbers of cells, in comparison to clusters in the middle-aged and aged SGZ (Fig. 5(A1–B3)). Quantification of the percentages of Sox-2⁺ cells expressing Ki67

demonstrated that 25% of Sox-2⁺ cells in the young SGZ expressed Ki67 (Fig. 5(E)). However, in the middle-aged SGZ, only 8% of Sox-2⁺ cells expressed Ki67, which decreased further to 4% in the aged SGZ (Fig. 5(E)). These results suggest that, though the numbers of Sox-2⁺ cells in the SGZ remain constant during aging, fractions of Sox-2⁺ cells that exhibit proliferation in the SGZ significantly decrease during the course of aging (68% decrease at middle age and 83% at old age). To validate these data further, we performed 5'-bromodeoxyuridine (BrdU) labeling experiments in all three age groups. We gave 4 injections of BrdU over a period of 18 h (one injection every 6 h), euthanized animals at 6 h after the last BrdU injection and examined Sox-2⁺ cells expressing BrdU using dual immunofluorescence and confocal microscopy (Fig. 5(C1–D3)). As observed with Ki67 analyses, all proliferating cells identified with BrdU were immunopositive for Sox-2 (Fig. 5(C1–D3)). The frequency and size of clusters of cells were generally greater in the young SGZ, in comparison to middle-aged and aged SGZ. Quantification of the percentages of Sox-2⁺ cells expressing BrdU in the SGZ revealed results that are similar to data obtained with Sox-2 and Ki67 analyses (Fig. 5(F)). Specifically, the percentage of Sox-2⁺ cells expressing BrdU in the SGZ was 15% at young age, which decreased to 2.5% at middle age (83% decline) and 1% at old age (93% decline; Fig. 5(F)). Thus, radically reduced percentages of Sox-2⁺ cells exhibit proliferation in the SGZ during middle age and old age, suggesting that aging of the SGZ is associated with greatly increased number of quiescent NSCs.

3.4. Number of GFAP⁺ astrocytes in the SGZ

The SGZ of the DG exhibited a large number of GFAP⁺ astrocytes in all three age groups (Fig. 6(A–C)). In most GFAP⁺ astrocytes, cell body or nuclear staining was not discernable though the position of soma was typically indicated by a junction where four to five thick GFAP⁺ processes emerge. To facilitate the identification of the position of soma, we employed neutral red counterstaining after GFAP immunostaining in this study. This counterstaining consistently demonstrated the presence of neutral red positive nucleus at or adjoining the junction where four to five thick GFAP⁺ processes emerge when examined using 100× objective lens (Fig. 6(D–F)). As fractions of astrocytes in the SGZ are likely NSCs, we quantified the total number of GFAP⁺ astrocytes in the SGZ using serial sections through the entire hippocampus. This demonstrated the presence of 62,600 astrocytes in the SGZ of young adult animals (mean ± S.E.M. = 62,600 ± 4057, *n* = 5; Fig. 6(G)). Interestingly, the middle aged and aged animals also exhibited comparable numbers of GFAP⁺ astrocytes in the SGZ (middle-aged, 57,800 ± 3760, *n* = 5; aged, 56,872 ± 4057, *n* = 5; Fig. 6(G)), suggesting that aging in the dentate SGZ is not associated with changes in the number of GFAP⁺ astrocytes.

3.5. Distribution and number of vimentin⁺ radial glial cells in the DG

As an additional measure of NSCs in the SGZ, we analyzed vimentin⁺ radial glial cells in the SGZ and the GCL of the DG, as previous studies imply that radial glial cells represent a type of NSCs and radial glia generate neurons and glia both in vitro and in vivo [16,56,57]. In addition, a recent study shows that radial glia-like cell bodies are involved in a one-to-one relationship with doublecortin⁺ newborn neurons in the adult DG [74]. In all age groups, vimentin⁺ cells were located in both upper and lower blades of the GCL with cell bodies located in either the SGZ or the lower half of the GCL (Fig. 7(A1–C2)). However, the density of vimentin⁺ cells was relatively greater in the lower blade. Typically, all vimentin⁺ cells exhibited one thick primary process emanating from the soma, which traversed radially through the GCL, bifurcated in the upper third of the GCL and branched in the dentate molecular layer (Fig. 7). In addition to this primary process, vimentin⁺ cells displayed a few thinner processes emanating from the soma which traversed the SGZ and the dentate hilus.

In the aged DG, majority of cells appeared to have profuse branching in the dentate molecular layer (Fig. 7(C1 and C2)).

To determine changes in the number of radial glia as a function of age, we quantified the absolute numbers of primary radial glial processes (i.e. the process that emanates from the soma and traverses the GCL) in the GCL (Fig. 7(D)). In young adult animals, the average number of vimentin⁺ primary radial glial processes in the GCL was 5814 (mean $\pm$ S.E.M. = 5814 ± 455 , $n = 5$; Fig. 7(D)). This number did not decrease with aging as both middle-aged and aged animals exhibited similar number of vimentin⁺ primary radial glial processes in the GCL (middle-aged, 5436 ± 582 , $n = 5$; aged, 5886 ± 392 , $n = 5$; Fig. 7(D)). Thus, aging does not alter the number of vimentin⁺ radial glia in the DG.

3.6. Volume fraction of RECA-1⁺ capillaries in the SGZ

The capillaries immunopositive for RECA-1 were found throughout the brain in all age groups. In the SGZ, these capillaries were mostly oriented parallel to the GCL (Fig. 8). However, some of the capillaries appeared to be extensions from those in the dentate hilus (Fig. 8(B2)). Interestingly, some of the capillaries in the aged dentate gyrus appeared wider in comparison to their counterparts in the young dentate gyrus (Fig. 8(A1–C2)). To determine whether aging alters one of the major components of vascular niches of neurogenesis in the SGZ [64], we quantified and compared the volume fraction of RECA-1⁺ capillaries in the SGZ of all age groups of rats (Fig. 8(D)). This demonstrated that RECA-1⁺ capillaries occupied 8% of the total SGZ volume in young adult animals, which was reduced to 5.9% in middle-aged animals (26% decrease, $p < 0.05$), and 5.7% in aged animals (29% decrease, $p < 0.05$; Fig. 8(D)). Thus, the amount of one of the major components of vascular niches decreases as a function of age in the SGZ.

3.7. Age-related alterations in the relationship between Sox-2⁺ cells and RECA-1⁺ capillaries in the SGZ

To determine whether decreased volume of RECA-1⁺ capillaries during aging would alter the location of putative NSCs, we examined Sox-2⁺ cells residing adjacent to RECA-1⁺ capillaries in the SGZ of young, middle-aged and aged animals (Fig. 9(A1–C3)). Quantification of the percentages of Sox-2⁺ cells residing adjacent to RECA-1⁺ capillaries demonstrated that 36% of Sox-2⁺ cells reside adjacent to RECA-1⁺ capillaries in the young SGZ (Fig. 9(D)). However, in the middle-aged and aged SGZ, decreased percentages (15–20%) of Sox-2⁺ cells resided adjacent to RECA-1⁺ capillaries (Fig. 9(D)). Thus, decreased volume of RECA-1⁺ capillaries within the SGZ during aging increases the distance between endothelial cells and the putative NSCs, which may in turn reduce the accessibility of NSCs to endothelial-cell derived neurotrophic factors.

4. Discussion

Our results provide novel evidence that aging does not decrease the number of Sox-2⁺ or GFAP⁺ cells in the dentate SGZ, a neurogenic region of the hippocampus where new neurons are added throughout life by NSCs. Moreover, the number of vimentin⁺ radial glia also remains stable in the dentate SGZ/GCL during the course of aging. Additionally, examination of the co-expression of other relevant proteins in Sox-2⁺ cells revealed that comparable fractions of Sox-2⁺ cells in the SGZ express GFAP, S-100, NG-2 and vimentin in young adult, middle-aged and aged groups indicating no age-related alteration in the phenotype of Sox-2⁺ cells. However, analyses of the proliferative status of Sox-2⁺ cells with Ki67 and BrdU labeling suggested an increased quiescence of NSCs as a function of age. Furthermore, the volume of RECA-1⁺ capillaries (one of the major components of vascular niches important for proliferation of NSCs) [65] within the SGZ was decreased in both

middle-aged and aged animals, in comparison to young adult animals. Consequently, the distance between NSCs and capillaries was increased in middle-aged and aged animals. Because Sox-2, GFAP, and vimentin are considered as putative markers of NSCs in the SGZ, these results collectively suggest that aging in the hippocampus is not associated with decreased number of NSCs in the SGZ. Thus, dramatically declined dentate neurogenesis observed during aging is not attributable to changes in the number or phenotype of NSCs in the SGZ. Rather, it appears to be an outcome of increased quiescence of NSCs due to age-related changes in NSC milieu, which likely include decreased volume of RECA-1⁺ capillaries observed in this study and decreased concentration of multiple NSC proliferation factors observed in earlier studies [37,76,77].

4.1. Specificity of Sox-2 as a marker of NSCs in the SGZ, and effects of aging on Sox-2⁺ cells in the SGZ

Precise detection and quantification of NSCs in the brain is difficult, apparently due to lack of markers that exclusively identify NSCs. However, recently, Sox-2 has been suggested as a marker of NSCs in the adult brain [6,20,32,66]. The specificity of Sox-2 expression in NSCs is supported by the observations that Sox-2 expression impedes neuronal differentiation and suppression of Sox-2 signaling leads to loss of progenitor marker proteins and initiation of neuronal differentiation [19,20,32,66]. Furthermore, a recent study using clonal analysis of Sox-2-EGFP⁺ cells demonstrate that multipotent NSCs isolated from both embryonic and adult brain consistently exhibit Sox-2 expression [6]. Other studies however demonstrate that Sox-2 is not only expressed in actively dividing NSCs in the neurogenic regions of the brain but also in a fraction of astrocytes [26,49]. Nevertheless, characterization of Sox-2⁺ cells with other relevant markers of glia and progenitors suggests that Sox-2 is a useful marker for studying the in vivo dynamics of NSCs in neurogenic regions when used in combination with appropriate cell-specific markers [49]. It has been proposed that proliferating Sox-2⁺ cells that lack GFAP expression are transit-amplifying neuronal precursor cells [49], Sox-2⁺ cells expressing nestin are putative stem cells, and Sox-2⁺ cells expressing S-100 β are local astrocytes in the SGZ [78]. Thus, although Sox-2 is not an exclusive marker of NSCs in the adult brain, quantification of Sox-2⁺ cells in combination with apt markers of glia and progenitors is useful for assessing changes in the number of NSCs in neurogenic regions of brain under conditions such as aging and disease.

Therefore, in this study, we not only quantified the absolute number of Sox-2⁺ cells in the entire SGZ but also characterized the potential changes in the phenotype of Sox-2⁺ cells using dual immunofluorescence for Sox-2 and markers of glial and/or progenitor cell specific proteins such as GFAP, S-100, NG-2 and vimentin. Our results clearly demonstrate that the number of Sox-2⁺ cells does not change in the SGZ between 4 and 24 months of age in F344 rats. Furthermore, it was seen that aging does not alter the fractions of Sox-2⁺ cells expressing different glial and/or progenitor cell specific proteins. In all age groups, 51–52% of Sox-2⁺ cells co-expressed GFAP (putative NSCs and local astrocytes), 8–9% co-expressed vimentin (radial glia with NSC properties), 32–35% co-expressed NG2 (putative NSCs and/or oligodendrocyte progenitors), and 28–34% contained S-100 β (local astrocytes). Taken together, these results imply that aging is not associated with decreased number or altered phenotype of NSCs in the SGZ.

4.2. Age-related changes in numbers of GFAP⁺ and vimentin⁺ cells in the SGZ

Numerous earlier studies suggest that new neurons in the adult neurogenic regions come from cells that exhibit characteristics of astrocytes and express GFAP [16,20,27,28,41,54,60,72,78]. Additionally, it has been shown earlier that isolated radial glial cells are capable of dividing and generating new neurons in neurogenic regions [23,56,57,59,64]. Furthermore, the observation that cell bodies of GFAP-expressing radial

glia exhibit one-to-one relationship with doublecortin⁺ newborn neurons in the adult DG supports the notion that radial glia represent a population of NSCs [74]. Therefore, we quantified the absolute numbers of GFAP⁺ astrocytes in the SGZ and vimentin⁺ radial glia in the SGZ and GCL as further measures of changes in NSC number with aging. Our results demonstrate that numbers of both GFAP⁺ astrocytes and vimentin⁺ radial glia remain stable in the SGZ between young adult age and old age, which is consistent with the data for Sox-2⁺ cells. Our finding of stable radial glial number during aging in male F344 rats however differs from a previous report for female F344 rats where a decreased number of nestin⁺ radial glia was observed with aging [62]. The discrepancy likely reflects differences in gender as well as the type of marker employed in the two studies. Thus, quantification of NSCs using additional putative markers such as GFAP and vimentin also suggests no alterations in NSC number with aging in male F344 rats.

4.3. Potential reasons for decreased neurogenesis during aging

Multiple previous reports demonstrate that dentate neurogenesis declines dramatically as early as middle age [52,53,62,71]. Furthermore, our recent study on different regulatory events of neurogenesis (i.e. production of new cells from NSCs, neuronal differentiation of newly born cells, migration and long-term survival of newly generated neurons) reveals that the major problem underlying the decreased neurogenesis during aging is the decreased production of new cells [68]. When taken together with our findings in this study that aging does not alter the number or phenotype of NSCs in the SGZ, it appears that NSCs in the SGZ of the aging hippocampus, though present in large numbers, exhibit considerably limited proliferation. Indeed, examination of the proliferative status of Sox-2⁺ cells in the SGZ using Sox-2 and Ki67 dual immunofluorescence reveals that fractions of Sox-2⁺ cells that exhibit proliferation in the SGZ significantly decrease during the course of aging (68% decrease at middle age and 83% at old age). Furthermore, quantification of the proliferating cells in the SGZ using BrdU labeling methods corroborated the findings of Sox-2 and Ki67 analyses (83% decrease at middle age and 93% decrease at old age). Thus, drastically diminished proportion of Sox-2⁺ cells display proliferation in the SGZ during middle age and old age, implying that hippocampal aging is associated with increased quiescence of NSCs in the SGZ.

Increased quiescence of NSCs may be linked to multiple age-related alterations in the milieu of the SGZ. Recent studies suggest that several factors that promote NSC proliferation alter considerably during aging. These include decreases in the concentration of: (i) neurotrophic factors such as the brain-derived neurotrophic factor (BDNF), the fibroblast growth factor-2 (FGF-2), the insulin-like growth factor-1 (IGF-1), and the vascular endothelial growth factor (VEGF) [76,77]; (ii) the phosphorylated cAMP response element binding protein and the neuropeptide Y [37]; (iii) cell death-derived stimulatory factors due to decreased rate of cell death in the GCL [39]; and (iv) the neurotransmitter serotonin [29,79]. Moreover, an elevated concentration of circulating corticosteroids observed during aging can also suppress the proliferation of NSCs [31,70]. Furthermore, other secretory factors capable of promoting dentate neurogenesis, such as neurogenesis-1, noggin, and Wnt protein [25,55,83] may also exhibit decline with aging. Our finding that aging does not alter the number of NSCs in the SGZ is also supported by the following observations in the previous reports. First, increased number of proliferating NSCs could be observed in the aging DG following direct intracerebral infusions of NSC proliferation factors such as the epidermal growth factor, FGF-2, or IGF-1 [43,53,79] or injections of NMDA receptor antagonists [62]. Second, NSC proliferation and neurogenesis in the aging DG can be restored by reducing corticosteroid levels [9]. Thus, a dramatic decline observed in the production of new neurons in the middle-aged and aged DG is mostly due to multiple age-related alterations in the milieu of the SGZ affecting the proliferation of the stem cells.

Nonetheless, a few modifications in NSCs themselves might also add to the age-related decline in dentate neurogenesis. This likely includes alterations in the self-renewal capability of NSCs with aging. Indeed, previous studies have revealed that NSCs from relatively older brains exhibit decreased telomerase levels [6] and telomerase shortening is linked to exhaustion of proliferative capability [35,38,73]. Additionally, an enhanced *in vivo* expansion of NSCs during the postnatal period via deletion of the cell cycle inhibitor, p21^{cip1/waf1} leads to impairments in long-term self-renewal and eventual exhaustion of NSCs in the aging mice, suggesting that adult NSCs have extensive, but only finite self-renewal capability [47]. Considering this, it is plausible that continued division during postnatal and adult periods likely alters the self-renewal capability of NSCs during aging via lengthening of cell cycle times and increased quiescence. Comparison of Sox-2 and BrdU data with Sox-2 and Ki67 results in this study does suggest lengthening of the cell cycle of NSCs with aging. This is because, in comparison to the percentage of Sox-2⁺ cells expressing BrdU (i.e. proliferating cells in the S-phase of the cell cycle), the percentage of Sox-2⁺ cells expressing Ki67 (i.e. proliferating cells in late G1, S, M and G2 phases of the cell cycle [15]) is 1.3-fold greater in the young SGZ. However, the ratio increases to 2.2-fold in the middle-aged SGZ and 3-fold in the aged SGZ. Thus, both increased quiescence and lengthening of cell cycle are observed in NSCs of middle-aged and aged SGZ. This may be an intrinsic regulatory mechanism within NSCs to allow their persistence through the lifetime of an organism [47].

4.4. Relationship between decreased vascular niches in the SGZ and dentate neurogenesis during aging

Adult neurogenesis in the SGZ occurs in close vicinity of vascular niches [65]. A recent study further suggests that ~32% of proliferating cells in the SGZ are associated with vascular niches [40]. Studies also suggest that neuro-genesis and angiogenesis run parallel, as both are regulated by modulating factors such as VEGF and its receptor *flk-1*, BDNF, and FGF-2 [40,63]. Measurement of the volume of RECA-1⁺ capillaries (one of the major components of vascular niches) in this study demonstrated 25–30% decrease in the overall vascular niches within the SGZ as a function of age. Consequently, the distance between endothelial cells and the putative NSCs was increased with aging. This was evidenced by decreases in the percentage of Sox-2⁺ cells residing adjacent to RECA-1⁺ capillaries with aging (44–58% decrease). Increased distance between endothelial cells and the putative NSCs may reduce the accessibility of NSCs to endothelial-cell derived neurotrophic factors [75]. The above results are not surprising considering the earlier findings that aging is associated with declined endothelial cell proliferation and reduced VEGF synthesis [87], and degenerative changes in endothelial cells and pericytes [84]. Since vascular niches in the SGZ are directly linked to neurogenesis, it appears that some of the age-related decline in dentate neurogenesis is due to decreases in vascular niches within the SGZ. This possibility is also supported by the observations that increased levels of VEGF, IGF-1, BDNF, and erythropoietin enhance neurogenesis via stimulation of angiogenesis and increased density of vascular niches [40,65,80,88]. Because endothelial cells secrete VEGF and the volume of RECA-1⁺ capillaries is reduced as a function of age, it is plausible that the aging SGZ has decreased concentration of VEGF. Indeed, a previous study reports that the concentration of VEGF in the entire hippocampus is decreased as early as middle age [76]. Because VEGF is an important regulator of dentate neurogenesis [11,24,33,34,42,81], decreased concentration of VEGF during aging (likely due to decreased proliferation of endothelial cells as revealed by reduced volume of RECA-1⁺ capillaries within the SGZ in this study) likely contributes to decreased neurogenesis during aging.

4.5. Conclusions

Our results clearly suggest that a dramatically declined dentate neurogenesis during aging does not reflect decreased number of NSCs in the SGZ with aging. Instead, it points to an increased quiescence and lengthening of cell cycle times in NSCs with aging likely due to multiple changes in NSC milieu. The milieu changes that contribute to NSC quiescence likely include decreased volume of RECA-1⁺ capillaries observed in this study and decreased concentration of multiple NSC proliferation factors observed in earlier studies [37,76,77]. These results support the idea that greater levels of NSC proliferation and neurogenesis are achievable in the aging DG via manipulations that make the microenvironment of the SGZ suitable for greater neurogenesis through increases in endothelial cell proliferation and concentration of neurotrophic factors such as VEGF, IGF-1, BDNF, and FGF-2. The most suitable manipulations likely include a combination of physical exercise, exposure to enriched environment, and new learning [12,45,51,86]. However, it remains to be deciphered whether continued application of these strategies is efficacious for maintaining increased proliferation of NSCs and increased neurogenesis all through life.

Acknowledgments

This research was supported by grants from the National Institute for Aging (NIH-NIA grant RO1 AG20924 to A.K.S.), the National Institute of Neurological Disorders and Stroke (RO1 NS 043507 to A.K.S.), and the Department of Veterans Affairs (VA Merit Review Award to A.K.S.).

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Fig. 1. Distribution of Sox-2 immunoreactive cells in the dentate subgranular zone (SGZ) of young (A1), middle aged (B1) and aged (C1) male F344 rats. A2, B2 and C2 are magnified views of the SGZ from A1, B1 and C1. Note that the density of Sox-2 immunoreactive cells appears similar across the three age groups. The bar chart (D) compares the absolute numbers of Sox-2 immunoreactive cells in the SGZ of young (YA) middle-aged (MA) and aged (AG) rats. Note that the numbers of Sox-2 immunoreactive cells do not change with aging. DH, dentate hilus; GCL, granule cell layer; ML, molecular layer. Scale bar, A1, B1, and C1 = 50 μ m; A2, B2, and C2 = 25 μ m.

Fig. 2. Sox-2 immunoreactive cells (in green color) in the subgranular zone (SGZ) of young and aged rats displaying different progenitor and glial cell markers (in red color), visualized by dual immunofluorescence and Z stacks using confocal microscopy. Examples include Sox-2 immunoreactive cells expressing GFAP (arrows in serial Z-sections, A1–J3), S-100β (arrows in K1–L3), NG2 (arrows in M1–N3). Arrowheads in all pictures denote Sox-2 immunoreactive cells lacking the expression of respective progenitor or glial cell markers. Scale bar, 25 μm.

Fig. 3.

Parts (A1–B3) illustrate Sox-2 immunoreactive cells in the sub-granular zone (SGZ) of young and aged rats displaying vimentin (arrows), visualized by dual immunofluorescence and confocal microscopic analysis. Parts (C1–C3) illustrate a SGZ area containing Sox-2 immunoreactive cells (in green color) and rip positive oligodendrocytes (in red color). Note that Sox-2 immunoreactivity is absent within the nucleus of rip positive oligodendrocytes. Arrowheads denote Sox-2 immunoreactive cells lacking the expression of rip. Parts (D1–F3) illustrate examples of SGZ areas containing Sox-2 immunoreactive cells (in red color) and doublecortin immunopositive newly born neurons (in green color), visualized by dual immunofluorescence and confocal microscopy. Sox-2 immunoreactivity is present in only the doublecortin positive new neurons exhibiting no or primitive dendrites (arrows in D1–D3). In contrast, doublecortin positive new neurons with longer vertical dendrites (relatively mature new neurons) clearly lack Sox-2 expression (E1–F3). Arrowheads denote examples of doublecortin immunoreactive cells lacking Sox-2 expression in the subgranular zone. Scale bar, 25 μ m.

Fig. 4. Percentages of Sox-2 immunoreactive cells in the subgranular zone (SGZ) of young, middle-aged and aged hippocampus expressing GFAP, vimentin, S-100 or NG2. Note that the fractions of Sox-2 immunoreactive cells expressing GFAP, vimentin, S-100 and NG2 remain stable during the course of aging.

Fig. 5.

Sox-2 immunoreactive cells in the subgranular zone (SGZ) of young and aged rats displaying Ki67 (A1–B3) or BrdU (C1–D3), visualized by dual immunofluorescence and Z-section analyses using confocal microscopy. Note that all proliferating cells (as identified by the expression of Ki67 or BrdU) are positive for Sox-2 (arrows). Arrowheads denote Sox-2 immunoreactive cells lacking Ki67 or BrdU. Scale bar, 25 μ m. Parts (E and F) illustrate the percentages of Sox-2 immunoreactive cells expressing Ki67 (E) and BrdU (F). Note that the percentages of Sox-2 immunoreactive cells expressing Ki67 or BrdU decline considerably during the course of aging. ** $p < 0.01$; *** $p < 0.001$.

Fig. 6. Distribution of GFAP⁺ astrocytes and their processes in the subgranular zone (SGZ) and granule cell layer (GCL) of young (A), middle aged (B) and aged (C) F344 rats. Parts (D–F) demonstrate the morphology of GFAP⁺ astrocytes encountered in the SGZ of young, middle-aged and aged rats, respectively. Asterisks denote nuclei of these cells. Scale bar, A–C = 50 μm; D–F, 10 μm. The bar chart in (G) illustrates the absolute number of GFAP⁺ cells within the SGZ of young (YA), middle-aged (MA) and aged (AG) rats. Note that, the numbers of GFAP⁺ astrocytes in the SGZ do not change with aging.

Fig. 7. Distribution and morphology of vimentin⁺ radial glial cells in the subgranular zone (SGZ) and granule cell layer (GCL) of young (A1) middle aged (B1) and aged (C1) F344 rats. A2, B2 and C2 are magnified views of vimentin⁺ radial glia from the SGZ/GCL of three age groups of animals. Morphologically, radial glial cells in all three age groups exhibit a thick primary process that traverses radially through the GCL, bifurcates in the outer third of the GCL and branches in the dentate molecular layer. In addition, these radial glia also exhibit a few thinner processes that traverse the SGZ. Scale bar, A1–C1 = 100 μ m; A2, B2, C2 = 25 μ m. The bar chart in (D) illustrates the absolute number of vimentin⁺ radial glia in the SGZ of young (YA), middle-aged (MA) and aged (AG) rats. Note that, the numbers of vimentin⁺ cells in the SGZ do not change with aging.

Fig. 8. Distribution of RECA-1 immunoreactive capillaries in the dentate subgranular zone (SGZ) and granule cell layer of young (A1), middle-aged (B1) and aged (C1) F344 rats. A2, B2 and C2 illustrate magnified views of RECA-1 immunoreactive capillaries (presumably the vascular niches) in the SGZ of three age groups of animals. Note that the density of capillaries in the SGZ of the young hippocampus is greater than that in the middle-aged and aged hippocampus. Additionally, the capillaries in the aged hippocampus appear relatively wider than capillaries in the young hippocampus. Scale bar, A1, B1, and C1 = 100 μm ; A2, B2, and C2 = 50 μm . The bar chart in (D) compares the volume fraction of RECA-1 immunoreactive capillaries/ μm^3 region of the SGZ between young (YA), middle-aged (MA) and aged (AG) groups. Note that, the overall volume of RECA-1 immunoreactive capillaries within the SGZ significantly decreases as early as middle age. GCL, granule cell layer. * $p < 0.05$.

Fig. 9.

Location of Sox-2 immunoreactive cells in relation to RECA-1 immunopositive capillaries in the subgranular zone (SGZ) of young (A1–A3), middle-aged (B1–B3) and aged (C1–C3) rats. Arrows denote Sox-2 immunoreactive cells that are located adjacent to RECA-1 immunopositive capillaries in the young, middle-aged, and aged SGZ. Arrowheads denote Sox-2 immunoreactive cells that are located away from the RECA-1 immunopositive capillaries. Scale bar, 25 μm. The bar chart in (D) compares the percentage of Sox-2 immunoreactive cells that are located adjacent to RECA-1 immunopositive capillaries in the SGZ of different age groups. Note that greater numbers of Sox-2 immunoreactive cells are located adjacent to RECA-1 immunopositive capillaries in the young SGZ, in comparison to both middle-aged and aged SGZ. * $p < 0.05$; *** $p < 0.001$.