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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-100β (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: 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 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

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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-100β (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 ($n = 5$/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, Carpenteria, 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