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Changes in hippocampal neurogenesis throughout early development☆

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ABSTRACT

Adult hippocampal neurogenesis drastically diminishes with age but the underlying mechanisms remain unclear. Here, age-related influences on the hippocampal early neuroprogenitor cell (NPC) pool was examined by quantifying changes in Sox1-expressing cells in the dentate gyrus subgranular zone from early adulthood (3 months) to middle age (12 months). Proliferation of distinct NPC subpopulations (Sox1+, Nestin+, and Doublecortin+) and newborn cell survival were also investigated. Examination of total 5-bromodeoxyuridine (BrdU)+ and Doublecortin (DCX)± cells revealed an early and dramatic age-dependent decline of hippocampal neurogenesis. Increasing age from 3 to 12 months was primarily associated with reduced total proliferation, in vivo (−79% of BrdU+ cells) but not in vitro, and DCX+ cell numbers (−89%). When proliferative rates of individual NPC subpopulations were examined, a different picture emerged as proliferating Nestin+ neuroprogenitors (−95% at 9 months) and BrdU+/DCX+ neuroblasts and/or immature neurons (−83% at 12 months) declined the most, whereas proliferating Sox1+ NPCs only dropped by 53%. Remarkably, despite greatly reduced proliferative rates and recent reports of Nestin+ neuroprogenitor loss, total numbers of early Sox1+ NPCs were unaffected by age (at least up to middle age), and newborn cell survival within the dentate gyrus was increased. Neuronal differentiation was concomitantly reduced; however, thus suggesting age-associated changes in fate-choice determination.

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1. Introduction

Hippocampal neurogenesis occurs throughout adulthood in the mammalian dentate gyrus (DG) as new neurons arise from neuroprogenitor cells (NPCs) in the subgranular zone (SGZ), the neurogenic niche between the hilus and granule cell layer (GCL) (Altman and Das, 1965; Cameron et al., 1993; Eriksson et al., 1998; Zhao et al., 2008). Production of new dentate granule neurons is a multistep process, regulated by extrinsic and local stimuli (Goldman and Chen, 2011). Approximately, 700 new cells are added the adult human hippocampus daily (Spalding et al., 2013). Interestingly however, an excess is generated, and only a fraction remains to differentiate into mature functional neurons and/or astrocytes,

depending on the need of the local hippocampal environment (Cameron et al., 1993; Encinas et al., 2011; Kuipers et al., 2009).

The number of neurons born postnatally declines rapidly with age (Klempin and Kempermann, 2007; Kuhn et al., 1996) and, in the mouse DG, only 8.5% of these are added after middle age (Lazic, 2012). This reduction represents one of the most conspicuous functional changes observed in the hippocampus across mammalian species. Mechanisms underlying this decline, although poorly understood, include permanent loss of neural stem cells (NSCs) and/or NPCs, their increased quiescence, impaired survival, and/or compromised neuronal fate commitment (Encinas et al., 2011; Hattiangady and Shetty, 2008; Kuhn et al., 1996; Lugert et al., 2010; McDonald and Wojtowicz, 2005; Olariu et al., 2007; Rao et al., 2006). Recently, a compelling argument was presented for a rapid and drastic depletion of Nestin+ progenitor pool as a key mechanism behind age-related decline of hippocampal neurogenesis (Encinas and Sierra, 2012). Here, in vivo and in vitro changes of hippocampal neurogenesis were explored through the early part of the developmental continuum (Coleman et al., 2004) using 3- to 12-month-old Sox1[eGFP] mice (Aubert et al., 2003).

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Conflicting reports have claimed a role for Sox1 expression in neural lineage commitment and differentiation (Kan et al., 2004; Pevny et al., 1998), as well as maintenance of the early NPC pool (Bylund et al., 2003; Elkouris et al., 2011; Suter et al., 2009; Zhao et al., 2004). A new model reconciling both scenarios has been proposed with Sox1 playing, on initial expression, a role in maintaining division within the early progenitor cell pool but, on continued expression, leading to neuronal differentiation (Kan et al., 2007). Sox1-cells have recently been identified as a subset of early NPCs (also described as radial astrocytes and type-1 cells) activated to produce new astrocytes and neurons (Venere et al., 2012). Notably, Sox1 expression precedes the appearance of Nestin, in line with the essential role of SoxB1 proteins, i.e. Sox1, in activating the Nestin enhancer (Tanaka et al., 2004). Analysis of changes in Sox1-expressing NPCs in the SGZ can therefore provide insight into whether age-related loss of NPCs is specific for Nestin+ NPCs or whether this also affects earlier lineages, to perhaps help reconcile seemingly contradictory findings of large numbers of early (Sox2-expressing) neuroprogenitors in aged hippocampi (Hattiangady and Shetty, 2008) with the progressive loss of Nestin+ cells (Encinas et al., 2011).

In this study, changes in total proliferating cells, the size of early neuroprogenitor pool, and numbers of total neuroblasts and/or immature neurons were investigated in young (3, 6, 9 months) and middle-aged (12 months) $Sox1^{eGFP}$ mice, by 5-bromodeoxyuridine (BrdU), Sox1, and Doublecortin (DCX) immunohistochemistry, respectively. Phenotypes of proliferating cells were also characterized by determining the fraction of BrdU+ cells coexpressing Nestin, Sox1, or DCX across the age groups (Kempermann et al., 2004; Zhao et al., 2008). Using NPCs isolated from 3- and 9-month-old hippocampi, we also examined their intrinsic in vitro proliferative "potential". Together, our results illustrate profound changes in hippocampal neurogenesis regulation between early adulthood and middle age. Notably, despite reported age-related loss of Nestin+ progenitor cells (Encinas et al., 2011), we only found a moderate and nonsignificant reduction in total early Sox1-expressing NPCs. Our findings indicate that the diminished production of new neurons in the aging hippocampus represents the result of multiple changes at different levels, which include lengthening of the neuronal differentiation process, changes in cell fate determination, and, most importantly, suppression of NPC proliferation. When changes in proliferation of discrete NPCs subpopulations were studied in detail, a more complex picture emerged, as proliferating Sox1+ cells declined by only 53% between 3 and 12 months, whereas Nestin+ neuroprogenitors already dropped by 95% at 9 months and BrdU+/DCX+ neuroblasts and/or immature neurons by 83% at middle age. Interestingly, increased NPC quiescence and/or lengthened neuronal maturation could thus represent adaptations to preserve new neurons' functions into old age. Our data also suggest that this sharp age-associated drop in NPC proliferation might be because of changes in the local neurogenic niche environment, as NPCs isolated from "young" and "older" hippocampi showed similar in vitro proliferative levels. These environmental changes, although detrimental for proliferation in vivo, seemed to promote survival, as greater fractions of surviving newborn cells were detected in middle-aged mice compared with younger animals.

2. Methods

2.1. Transgenic animals and experimental design

Transgenic homozygous $Sox1^{eGFP}$ (129xMF1) F1 mice, acquired from the University of Edinburgh (a gift from Prof. Austin Smith), were generated by inserting the enhanced GFP (eGFP) reporter into the Sox1 gene via gene targeting (Ying et al., 2003). The GFP knock-in allows visualization of Sox1 expression by immunohistochemistry (Aubert et al., 2003). Male mice of different ages (3, 6, 9, and 12 months) were individually housed with ad libitum access to food and water and maintained on a 12-hour light-dark cycle (light on at 7 AM). Animals received at least 2 weeks to acclimatize to their new environmental conditions before onset of the experiment. This study was designed to minimize the use of animals and was carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), the guidelines of the United Kingdom Animals (Scientific Procedures) Act (1986) and conformed to GlaxoSmithKline ethical standards.

$Sox1^{eGFP}$ mice were randomly assigned to 8 groups across 2 experiments:

- Aging effects on cell proliferation: 3-, 6-, 9-, and 12-month-old mice (n = 5/group) received 2 injections of BrdU (100 mg/kg each) 4 and 2 hours before sacrifice.
- Aging effects on newborn cell survival: 3-, 6-, 9-, and 12-month-old mice (n = 5/group) received 2 injections of BrdU (2 hours apart; 100 mg/kg each) 14 days before sacrifice. As one of the main goals of this experiment was to examine the influence of increasing age on the incorporation of newborn cells into the existing circuitry, this 2-week survival period was chosen based on reports that most newborn cells die during the first 7–10 days post-BrdU injection (Kuipers et al., 2009; Snyder et al., 2001) whereas most of those that survive this critical period differentiate into neurons or glial cells. This 2-week survival period also maximized numbers of BrdU+/DCX+ cells (Brown et al., 2003). As another aim was to investigate age-related changes in numbers of BrdU+/DCX+ neuroblasts and immature neurons, this longer survival period also allowed more BrdU+ cells to fully differentiate into mature neurons resulting in the cessation of DCX expression and, ultimately, a reduction of the fraction of double-labeled BrdU+/DCX+ cells.

2.2. Histologic procedure

Mice were sacrificed with an overdose of sodium pentobarbital preceding a transcardial perfusion with 20 mL of 0.1 M sodium phosphate buffer (PBS, pH 7.4). Brains were removed and postfixed for 7 days at 4 °C in 4% paraformaldehyde (in 0.1 M PBS, pH 7.4) before being transferred and stored in PBS with 0.1% sodiumazide at 4 °C. Following cryoprotection by overnight immersion in 30% sucrose, coronal serial sections of 40 μm were prepared on a cryostat microtome. Sections were collected in PBS containing 0.1% sodiumazide and stored at 4 °C. Immunohistochemical stainings (brightlight microscopy) were performed using coronal sections (every 6th section throughout the hippocampus). Additional sections were processed for confocal analysis (every 12th section throughout the hippocampus) through 2 triple immunostainings: BrdU-GFP-Nestin and BrdU-GFP-DCX.

2.2.1. Sox1 immunohistochemistry

For GFP immunohistochemistry, use was made of a goat anti-GFP primary antibody (Abcam ab6673; 1:5000 dilution in 0.1 M PBS, pH 7.4; 48-hour incubation) and a biotinylated rabbit anti-goat secondary (Vector Laboratories Ltd, Peterborough, UK; 1:750 dilution in 0.1 M PBS, pH 7.4; 2-hour incubation). This was followed by incubation in the avidin-biotin complex (ABC) kit (for 2 hours) and visualization of the reaction product using diaminobenzidine (DAB) as chromogen with H_2O_2 for 15 minutes. Sections were washed, mounted on slides, dehydrated, and coverslipped with DPX (Fig. 1A–L).
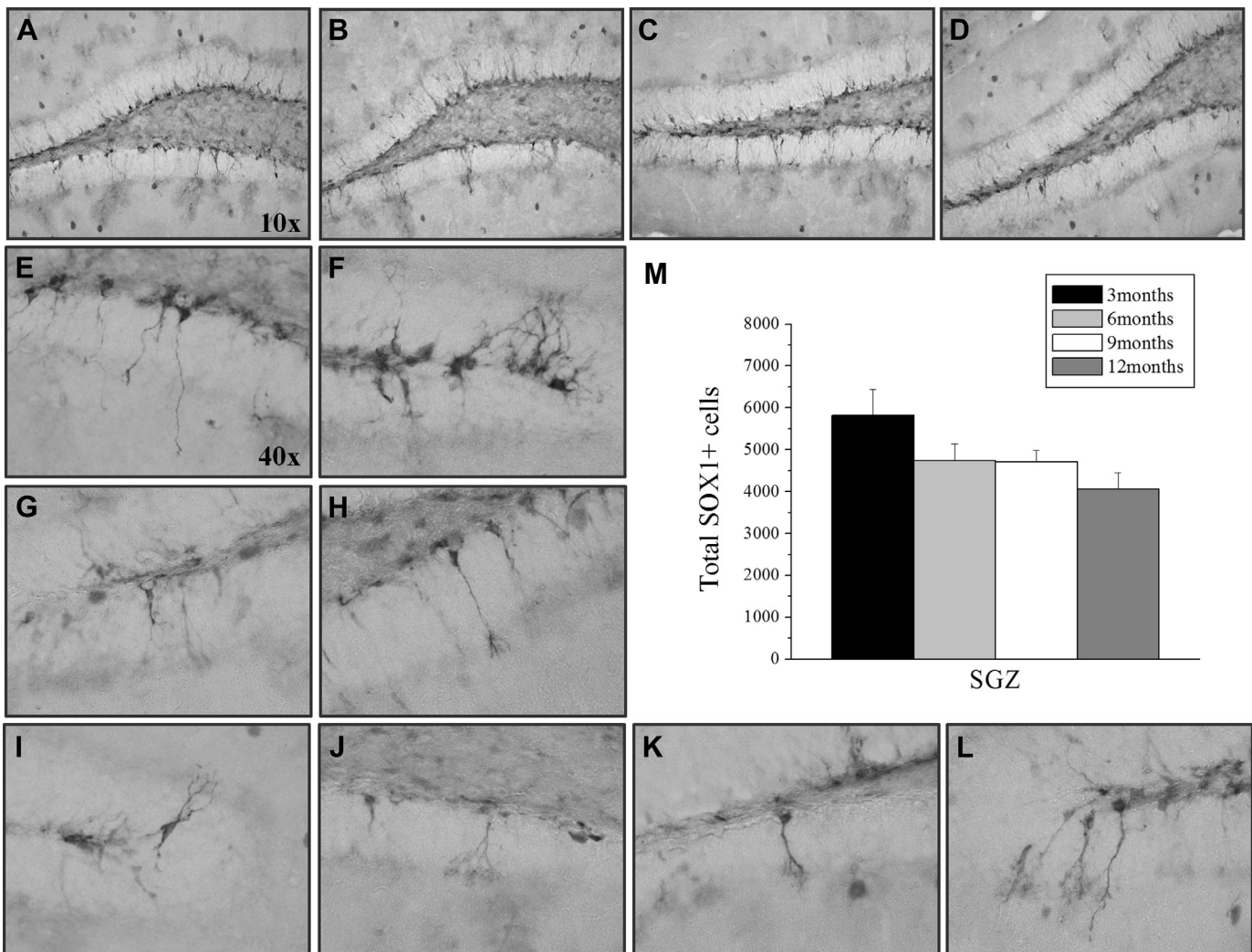
Fig. 1. Sox1/GFP labeling in the hippocampal dentate gyrus. Representative photomicrographs depicting Sox1(GFP) immunoreactivity across the age groups (10×): 3 months (A), 6 months (B), 9 months (C), and 12 months (D). As expected, most of Sox1+ cells were located in the SGZ, where adult NPCs reside. High power photomicrograhps (40×) illustrating different morphologies of Sox1-expressing cells (E–L). (M) The influence of increasing age on total numbers of SGZ Sox1-labeled cells. From early adulthood to middle age, a moderate and nonsignificant reduction was found on total Sox1+ cells in the SGZ of the DG ($p = 0.079$). Abbreviations: DG, dentate gyrus; NPCs, neuroprogenitor cells; SGZ, subgranular zone.

2.2.2. BrdU injections and immunohistochemistry

To label dividing cells, animals were given BrdU (2 intraperitoneal injections, 100 mg/kg each, 2 hours apart; Sigma Aldrich, UK). As a thymidine analog, BrdU incorporates into DNA of dividing cells during the S-phase of the cell cycle (which lasts approximately 8 hours) and has a postinjection bioavailability estimated at 2 hours. Immunostaining for BrdU was used to determine the number of proliferating cells and their progeny. BrdU immunohistochemistry was performed using the ABC method as previously described (Kuipers et al., 2009, 2013). In short, BrdU labeling requires the following pretreatment: DNA denaturation (50% formamide/2xSSC pH 7.0, 120 minutes at 65 °C) and acidification (2 M HCl, 30 minutes at 37 °C). Primary antibody was a rat monoclonal anti-BrdU antibody (AbD Serotec, MCA2060; 1:1000 dilution in 0.1 M PBS, pH 7.4; overnight incubation) whereas secondary antibody was a biotinylated rabbit anti-rat (Vector Laboratories Ltd; 1:750 dilution in 0.1 M PBS, pH 7.4; 2-hour incubation). Sections were subsequently incubated with the Vector ABC kit (Vector Laboratories Ltd; 2-hour incubation). The reaction product was visualized by adding DAB and H_2O_2 for 7 minutes (Vector DAB kit, Vector Laboratories Ltd). Finally,

sections were washed, mounted on slides, dehydrated, and coverslipped with DPX (Fig. 2C–G).

2.2.3. Doublecortin immunohistochemistry

Doublecortin immunostaining was performed using the ABC method. Primary antibody was a goat anti-DCX (Santa Cruz Biotechnology; SC-8066; 1:200 dilution in 0.1 M PBS, pH 7.4; 48-hour incubation), whereas secondary antibody was a biotinylated rabbit anti-goat (Vector Laboratories Ltd; 1:750 dilution in 0.1 M PBS, pH 7.4; 2-hour incubation). Sections were subsequently incubated with the Vector ABC kit. The reaction product was visualized by adding DAB and H_2O_2 for 10 minutes. Finally, sections were washed, mounted on slides, dehydrated, and coverslipped with DPX (Fig. 3A–L).

2.2.4. Quantification of total BrdU, Sox1, and DCX labeling

For quantification of BrdU+, DCX+, or Sox1(GFP)+ cells, every sixth section (240 μm apart) throughout the rostral and/or caudal extent of the hippocampus was collected and coded before immunohistochemical analysis to ensure objectivity. All labeled cells in the SGZ (2-cell-thick region along the inner GCL border) and/or GCL were counted (at 20×) regardless of size or shape. Cells were
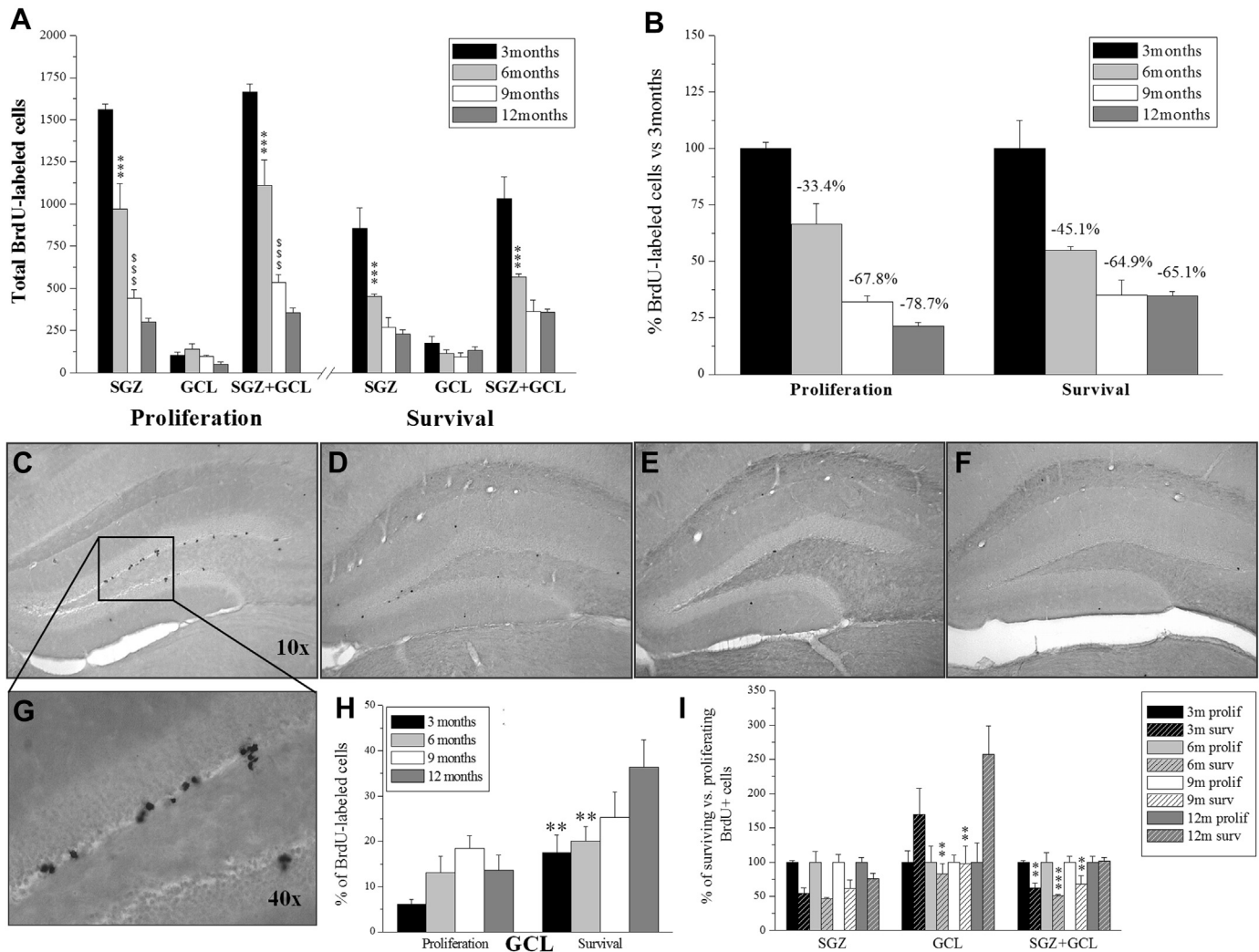
Fig. 2. BrdU labeling in the hippocampal dentate gyrus. Aging was associated with a progressive decline of total proliferating (BrdU+) cells in the SGZ (A, proliferation) consisting of a 37.8% drop between 3 and 6 months of age (*** $p < 0.001$), 54.6% between 6 and 9 months of age ($\$\$\$\ p < 0.001$), and 31.2% between 9 and 12 months of age ($p > 0.05$). Although not significant, a decrease in BrdU+ cells also occurred in the GCL. Together, a significant age-related reduction occurred in the DG (SGZ + GCL). Total BrdU+ cells dropped by 33.4% between 3 and 6 months (*** $p < 0.001$), 51.7% between 6 and 9 months ($\$\$\$\ p < 0.001$), and 33.9% between 9 and 12 months ($p > 0.05$). Aging was also associated with a reduction of total BrdU+ surviving cells in both the SGZ and DG (A, survival), dropping respectively 47.2% and 45.1% between 3 and 6 months (*** $p < 0.001$), 40.2% and 36% between 6 and 9 months ($p > 0.05$) and 15% and 0.7% between 9 and 12 months ($p > 0.05$). Compared with 3 months, total DG BrdU+ cells dropped 33.4% by 6 months ($p < 0.001$), 67.8% by 9 months ($p < 0.001$), and 78.7% by 12 months of age ($p < 0.001$) (B, proliferation), whereas 2-week-old BrdU+ cells declined by 45.1% ($p < 0.001$), 64.9% ($p > 0.05$), and 65.1% by 6, 9, and 12 months, respectively ($p < 0.001$) (B, survival). Representative photomicrographs (10×) depicting BrdU labeling in newborn DG cells at 3 months (C), 6 months (D), 9 months (E), and 12 months (F). Panel G represents a blowout image (40×) of the squared area in panel C and illustrates BrdU+ cells clusters commonly observed in the SGZ at 3 months. At 6–12 months clusters disappeared and BrdU+ cells appeared as isolated cells. (H) The percentages of BrdU+ cells in the GCL during the proliferation and survival experiment. As newly generated cells migrate from the SGZ to the GCL where they mature, the relative amount of BrdU+ cells in the GCL of animals sacrificed 14 days following BrdU administration was significantly higher than in those sacrificed after 4 hours ($p < 0.001$). This effect was particularly evident in 12-month-old mice which showed increased percentages of GCL newborn cells compared with 3 (** $p = 0.002$) and 6 months (** $p = 0.006$). Finally, aging resulted in a gradual increase of BrdU+ cell survival. (I) The "2-week survival rate", a measure reflecting the impact of aging on the survival of newborn DG cells. Results revealed an increased percentage of 2-week-old BrdU+ cells in the 12-month-old DG compared with 3 (** $p = 0.0027$), 6 (*** $p = 0.00035$), and 9-month-old animals (** $p = 0.0079$), largely because of a 2.5-times increased survival rate in the GCL (** $p = 0.0015$ vs. 6 months; ** $p = 0.003$ vs. 9 months). Abbreviations: BrdU, 5-bromodeoxyuridine; DG, dentate gyrus; GCL, granule cell layer; SGZ, subgranular zone.

considered subgranular if they were in or adjacent to the SGZ. Cells located more than 1 cell width away were considered granular. Cell clusters were examined under the 40× objective. Total numbers of BrdU+, Sox1+, and DCX+ cells in the SGZ, GCL, and dentate gyrus (sum of cell in the SGZ and GCL) were estimated by multiplying the total number of cells every sixth section by 6 and reported as mean ± SE (Kuipers et al., 2009, 2013). Although this method does not account for split nuclei and a potential slight overcount, the thickness of the sections combined with the small object width and large intergroup differences, render the influence of this error as minimal and likely of no consequence to the conclusions.

We further distinguished DCX+ cells into morphologic subclasses based on an adaptation of the stages of neuronal differentiation described previously (Plümpe et al., 2006). Class I cells constitute younger cells located in the SGZ and without a dendrite or with short processes reaching no further than the granule cell layer, whereas class II cells represent the most mature DCX+ cells, characterized by a primary dendrite orientated perpendicular to the SGZ and projecting radially up into the molecular layer (Van Bokhoven et al., 2011).

2.2.5. Immunofluorescent multilabeling

For simultaneous BrdU/GFP/Nestin or BrdU/GFP/DCX triple labeling, every 12th section was processed. Free-floating 40-μm-thick sections were pretreated with 2 N HCl for 30 minutes and incubated for 48 hours with rat monoclonal anti-BrdU (1:500), goat polyclonal anti-GFP (1:1000) and mouse monoclonal anti-Nestin (1:200; BD

Biosciences 611659) or with rat monoclonal anti-BrdU (1:500), rabbit polyclonal anti-GFP (1:500; Abcam ab290) and goat polyclonal anti-DCX (1:200). Sections were then incubated for 6 hours with Alexa 488-, Alexa 555-, and Alexa 647-conjugated secondary antibodies against the appropriate species (1:200; Invitrogen) and coverslipped using Vectashield mounting medium (Vector Laboratories Ltd) (Fig. 4A and B).

Fluorescently labeled cells were imaged on a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems, Heidelberg GmbH) equipped with 3 lasers (Argon 488, Krypton 568, and HeNe 633). Care was taken to verify double labeling and control for false positives by analyzing BrdU-positive nuclei in their z-axis and rotating them in orthogonal x-y planes using a $40\times$ objective (1.5 µm steps). To exclude potential cross-bleeding between detection channels, triple immunohistochemical stainings were imaged in sequential scanning mode.

2.3. In vitro procedures

2.3.1. Dissection and preparation of NPC cultures
NPCs were isolated from the hippocampal SGZ of 3- and 9-month-old mice. Mice were sacrificed with a sodium pentobarbital overdose, and brains were rapidly removed. Under a dissection microscope, hemispheres were parted and hippocampi were rolled out toward the edge. Isolated hippocampi were transferred to Hank Balanced Salt Solution and dissociated in the salt solution containing trypsin (0.25%), dispase I, and DNAse for 5 minutes at 37 °C. After further mechanical dissociation (trituration) with a fire polished Pasteur pipette, the enzymatic reaction was stopped by the addition of 5 volumes of basic Neurobasal-A medium (NB-A) supplemented with B27 without vitamin A (Invitrogen). Cells were pelleted by gentle centrifugation and the medium aspirated. Cells were resuspended in propagation medium (NB-A containing B27, recombinant human EGF, and recombinant human FGF2; 10 ng/mL each; R&D Systems) and transferred to uncoated plastic flasks to allow formation of neurospheres over 5–7 days. Neurospheres were harvested by centrifugation, washed and seeded in propagation medium supplemented with Matrigel basement membrane (BD Biosciences; 2 µg/mL added to cold medium) to induce adherence to the plastic. Once adhered, NPCs migrate and form monolayered colonies. These were dissociated and harvested by centrifugation (1000 rpm for 2 minutes). The pellets were resuspended and cells mechanically dissociated by repeated pipetting followed by transfer to fresh propagation medium supplemented with Matrigel and thereafter propagated in uncoated plastic flasks as a monolayer. NPCs underwent rapid expansion (Fig. 5A) and every 3–4 days, at 70% confluence, were passaged 1:10 to 1:20. NPCs were used for experiments up to passage 20 (Goffin et al., 2008).

2.3.2. Immunocytochemical characterization
NPCs were seeded into 96-well plates at a density of 5000 cells/well and cultured for 24 hours. Cells were then fixed using FixDenat solution (Roche Molecular Biochemicals) for 30 minutes at 20 °C and immediately processed for immunocytochemistry. Fixed cells were washed twice in PBS and incubated with an anti-musashi1 antibody (1:200) or anti-SOX2 antibody (1:200) diluted in PBS containing Tween (0.1%) and BSA (3%). After overnight incubation at 4 °C, cells were washed in PBS and incubated with the specific secondary fluorescent Alexa 594 antibody (1:1000, 1 µg/mL, Molecular Probes) diluted in PBS/Tween/BSA for 1 hour at room temperature before 2 final rinses with PBS (Fig. 5B and C).

2.3.3. BrdU incorporation and immunocytochemistry
NPCs were seeded into 96-well plates at a density of 5000 cells/well and cultured for 24 hours. Cells were washed twice with fresh medium containing EGF and bFGF (10 ng/mL each) and incubated for 24 hours, after which BrdU (5 µM, final concentration) was added for 1, 5, 15, 30, 60, or 240 minutes. Cells were fixed with FixDenat solution (Roche Molecular Biochemicals) for 30 minutes at 20 °C and immediately processed for immunocytochemistry. Fixed cells were washed twice in PBS and incubated with a primary rat anti-BrdU antibody (1:2000, 1 µg/mL, Serotec) diluted in PBS with Tween (0.1%) and BSA (3%). After overnight incubation at 4 °C, cells were washed in PBS and incubated with the secondary anti-rat Alexa 594-conjugated antibody (1:1000, 1 µg/mL, Molecular Probes) and Hoechst 33,342 dye (1:10,000, 1 µg/mL, Invitrogen) diluted in PBS/Tween/BSA for 1 hour at room temperature before 2 final rinses with PBS and analysis using ArrayScan II (Molecular Devices) (Fig. 5D). BrdU incorporation was quantified by capturing single images and expressed as the percentage of BrdU-positive cells compared with Hoechst-labeled nuclei. All data were averaged from 16 images per treatment performed in triplicate from a minimum of 3 independent experiments.

2.4. Statistics

SigmaStat 3.1 software was used to perform analysis of variance. Results were analyzed by 1-way or 2-way analysis of variance. Results were considered significant when $p \leq 0.05$. Pairwise multiple comparison procedures (Holm-Sidak post-hoc method) were applied to more accurately assess the source of variation between groups.

3. Results

3.1. Total Sox1+ cells

Sox1 is a transcription factor in the SoxB1 subgroup which also includes Sox2 and Sox3. These proteins are crucial for central nervous system development and continue to be expressed throughout adulthood in self-renewing NPCs in the subventricular zone (SVZ) and DG (Wegner and Stolt, 2005). Together, they regulate NPC identity by promoting proliferation and influencing neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Pevny et al., 1998). New findings suggest that prolonged Sox1 expression eventually results in initiation of the neuronal differentiation cascade (Kan et al., 2007).

Functions of individual SoxB1 members are difficult to ascertain as these proteins compensate for each other's loss, suggesting a high degree of functional redundancy (Ekonomou et al., 2005; Graham et al., 2003; Kan et al., 2007). Yet, despite overlapping structure and functions, Sox1, Sox2, and Sox3 seem to have distinct roles in neural specification and/or differentiation (Archer et al., 2011). From a temporal perspective, it is difficult to accurately position Sox1 expression in relation to other endogenous markers, for example, Sox2, Nestin, or GFAP because of overlapping expression patterns of Sox genes and differences in the regulation of neurogenesis during embryonic development and adulthood. Although these genes are all expressed very early during adult hippocampal neurogenesis (by type-1 and/or 2a cells) (Fig. 4E), their functions are dose-dependent and, therefore, absolute protein levels and/or relative abundance may identify different NPCs subpopulations. Elevated GFAP and Sox2 levels characterize undifferentiated and multipotent ("true" type-1) NPCs in the SVZ and SGZ. Sox2+ NPCs isolated from the adult brain can be propagated in culture where they maintain the ability to differentiate into neurons, astrocytes, and oligodendrocytes (Ellis et al., 2004), and their self-renewal and differentiation capacities were also confirmed in vivo (Suh et al., 2007). Specific conditional Sox2 deletion induces a rapid loss of Nestin+ NPCs and a decline in dentate cell proliferation,
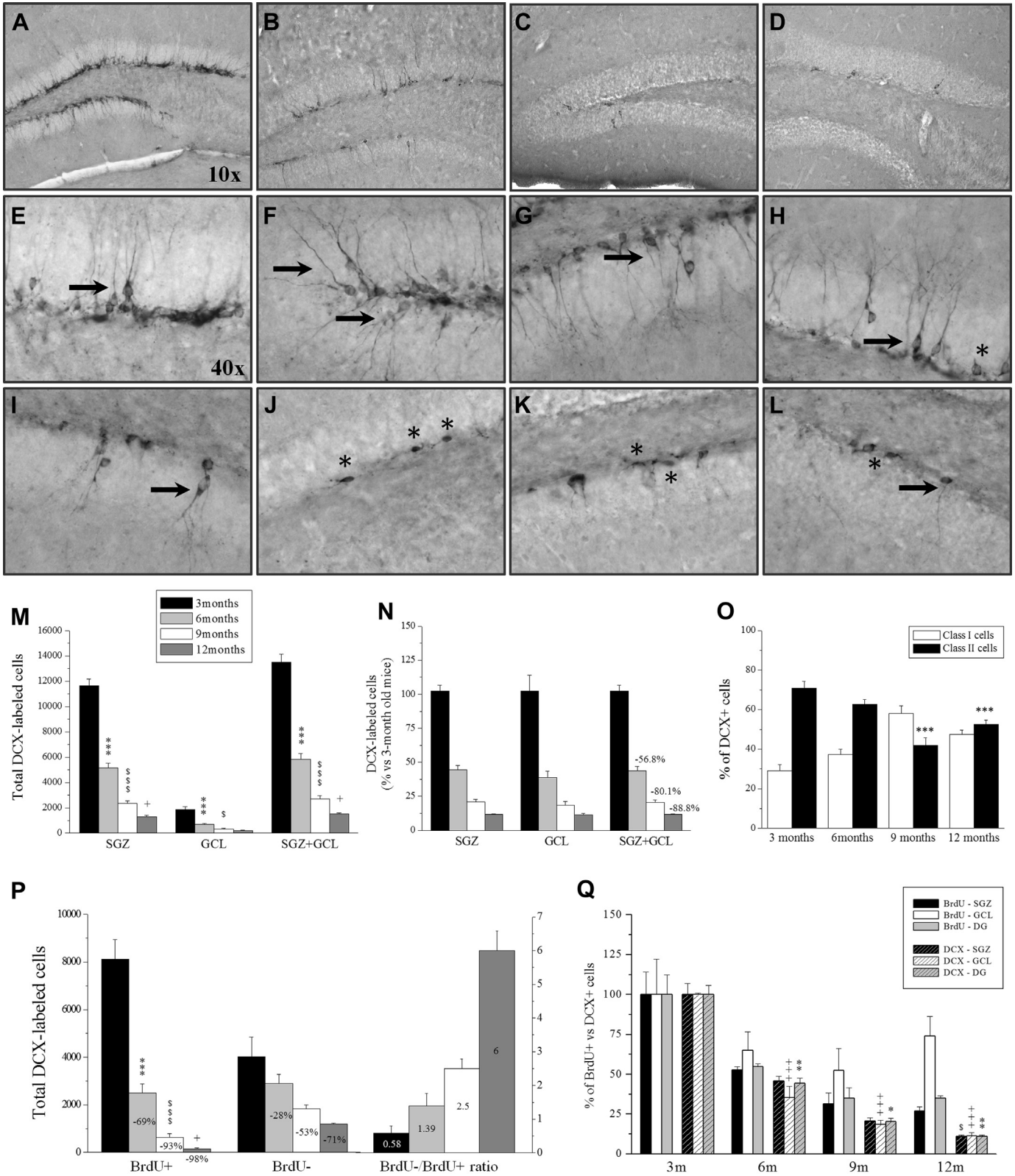
Fig. 3. Doublecortin labeling in the hippocampal dentate gyrus. Representative photomicrographs (10×) depicting DCX immunoreactivity at 3 months (A), 6 months (B), 9 months (C), and 12 months (D). DCX is a specific marker for neuroblasts and immature neurons born predominantly during the previous 12 days. In all age groups, most of the DCX+ cell bodies were located in the SGZ or the inner third layer of the GCL. High-power images (40×) of DCX+ cells with different morphology (E–L). Examples of class I cells (asterisk) with short processes (often parallel to the SGZ) reaching no further than the GCL. Examples of class II cells (arrow) with at least 1 dendrite reaching into the molecular layer and occasionally showing delicate branching with few major branches. (M and N) The influence of aging on the total numbers and percentages of DCX-labeled cells, respectively. An aging-associated reduction of total DCX+ cells was particularly evident between 3 and 9 months. In the SGZ, GCL, and DG, DCX+ cells decreased respectively by 55.7% (*** $p < 0.001$), 63.6% (*** $p < 0.001$), and 56.7% ($p < 0.001$) at 6 months; 54.5% ($\$\$\$$ $p < 0.001$), 50.3% ($\$$ $p = 0.048$) and 54.5% ($\$\$\$$ $p < 0.001$) at 9 months; and 44.2% (+ $p = 0.037$), 40% ($p > 0.05$), and 43.7% (+ $p = 0.047$) at 12 months. Compared with 3-month-old mice, DG DCX expression was reduced by 56.8%, 80.1%, and 88.8% by 6, 9, and 12 months ($p < 0.001$) (N). (O) The

substantiating the crucial role of Sox2 in maintaining undifferentiated NPCs and hence neurogenesis in the adult hippocampus (Favaro et al., 2009; Ferri et al., 2004). Although Sox1 has also been documented in type-1 cells, its expression follows that of GFAP and Sox2 with a more restricted pattern, labeling only a subset of GFAP+ radial astrocytes and Sox2+ nonradial cells within the SGZ (Venere et al., 2012). Importantly, Sox1+ NPCs are responsible for producing most of the neuroblasts and, ultimately, adult-born new neurons. Increased Sox1 expression (accompanied by a reduction in GFAP and Sox2) have been suggested to act at an early (perhaps earliest) step, of the neuronal differentiation program, labeling more restricted NPCs than Sox2+ and GFAP+ cells. With regard to Nestin, Sox1 expression precedes the appearance of Nestin, as Sox1 directly activates Nestin-enhancer elements inducing the expression of this intermediate filament protein (Kan et al., 2004; Tanaka et al., 2004) (Fig. 4E).

Our results indicate that most of the Sox1+ cells were confined to the inner SGZ (Fig. 1A–D) and demonstrated an astrocytic morphology (Fig. 1I–L). Total numbers of Sox1-expressing cells dropped from circa 5800 ($\pm$600) in 3-month-old mice to circa 4700 ($\pm$400) in 6-month-old animals (-18.6%). This number remained stable until 9 months (4700 $\pm$ 300) and gradually declined to circa 4000 in 12-month-old mice (-13.7% compared with 9 months). Together, increasing age from 3 to 12 months was associated with a moderate, yet nonsignificant, reduction in total numbers of Sox1-expressing cells in the DG SGZ (-30.2%: $p = 0.079$) (Fig. 1M; Table 1).

3.2. Total BrdU+ cells

3.2.1. In vivo proliferation experiment: total proliferating cells

Aging-dependent effects on total proliferating cell were examined in the DG of 3-, 6-, 9-, and 12-month-old mice by administering BrdU shortly before sacrifice. Although at 3 months, BrdU+ cells were often found grouped in small clusters (Fig. 2C and G) they appeared as isolated individual cells in older animals (Fig. 2D–F).

An age-dependent decline in total BrdU+ cells was observed in the SGZ ($p < 0.001$) (Fig. 2A; proliferation) consisting of a 37.8% drop between 3 and 6 ($p < 0.001$), 54.6% between 6 and 9 ($p < 0.001$), and 31.2% between 9 and 12 months of age ($p > 0.05$). Although not significant, a decrease occurred in the GCL ($p = 0.053$) and together with the SGZ, a significant age-related reduction was apparent in the DG (SGZ + GCL; $p < 0.001$). Compared with 3-months, DG BrdU+ cells dropped 33.4% by 6 months ($p < 0.001$), 67.8% by 9 months ($p < 0.001$), and 78.7% by 12 months of age ($p < 0.001$) (Fig. 2B; proliferation), numbers consistent with reports from other rodent studies (Bondolfi et al., 2004; Kuhn et al., 1996; Lazic, 2012; Rao et al., 2005). As this decrease constitutes 33.4% during early adulthood (3–6 months; $p < 0.001$), 51.7% during mid-adulthood (6–9 months; $p < 0.001$), and 33.9% in middle age (9–12 months; $p > 0.05$), the strongest drop in DG cell proliferation thus occurred during mid-adulthood (from 6 to 9 months) (Table 1).

3.2.2. In vivo survival experiment: total surviving cells

To determine age-dependent effects on newborn cell survival, changes in total surviving BrdU+ cells were examined in 3-, 6-, 9-, and 12-month-old mice that received BrdU 14 days before sacrifice and were then left undisturbed. Unlike the proliferation experiment, no BrdU+ cell clusters were observed in any age group and most labeled cells appeared isolated and spread out along the SGZ and/or GCL border. Many also displayed a punctuate pattern of BrdU staining.

Aging significantly decreased total numbers of 2-week-old BrdU+ cells in the SGZ ($p < 0.001$) (Fig. 2A; survival) dropping 47.2% between 3 and 6 months ($p < 0.001$), 40.2% between 6 and 9 months ($p > 0.05$), and 15.0% between 9 and 12 months of age ($p > 0.05$). There was also a slight, yet insignificant, decrease in the GCL ($p > 0.05$), yielding a significant age-dependent decline of cell survival in the DG (SGZ + GCL; $p < 0.001$). Two-week-old BrdU+ cells were reduced by 45.1% ($p < 0.001$), 64.9% ($p > 0.05$), and 65.1% by 6, 9, and 12 months, respectively ($p < 0.001$) (Fig. 2B; survival), constituting the greatest drop of 45.1% during early adulthood (3–6 months) ($p < 0.001$), 36.0% during mid-adulthood ($p > 0.05$), and 0.7% during middle age ($p > 0.05$) (Table 1).

3.2.3. Migration of BrdU+ cells toward the GCL and "2-week survival rate"

New cells generated in the SGZ migrate to the GCL where they differentiate into mature granule neurons in response to local cues. To evaluate effects of age on migration and survival rates, we calculated the relative fraction of BrdU+ cells in the SGZ and GCL for each animal, using the following equations:

- Percentage of BrdU+ cells in the SGZ = (SGZ$_{total\ BrdU+\ cells}$/ [SGZ$_{total\ BrdU+\ cells}$ + GCL$_{total\ BrdU+\ cells}$]) $\times$ 100.
- Percentage of BrdU+ cells in the GCL = (GCL$_{total\ BrdU+\ cells}$/ [SGZ$_{total\ BrdU+\ cells}$ + GCL$_{total\ BrdU+\ cells}$]) $\times$ 100.

As expected, percentages of BrdU+ cells in the GCL of animals sacrificed 2 weeks after BrdU administration were significantly higher than those of animals sacrificed after 4 hours (proliferation versus survival, $p < 0.001$) (Fig. 2H). This is not surprising because surviving BrdU+ cells had 2 more weeks to migrate to the GCL. The percentage of newborn cells in the GCL also increased gradually with age ($p = 0.011$), mostly because of increased percentages of 2-week-old BrdU+ cells (survival experiment), particularly in the oldest animals. Twelve-month-old mice demonstrated significantly higher percentages of surviving BrdU+ cells compared with both 3- ($+108\%$, $p = 0.002$) and 6-month-old mice ($+81.3\%$ $p = 0.006$) (Fig. 2H).

We then calculated the survival rates of BrdU+ cells by normalizing data from the survival experiment with that of the proliferation experiment. This resultant "2-week survival rate" provided a measure of the impact of age on newborn cell survival (Fig. 2I). Results revealed a significant age-related effect on relative newborn cell survival, reflected by increased percentages of 2-week-old BrdU+ cells in the GCL ($p = 0.006$) and DG (SGZ+GCL: $p = 0.002$)

influence of progressing age on class I and II cell fractions. In young mice (3 and 6 months), a large percentage of DCX+ cells (71% and 63%, respectively) showed vertically oriented dendrites extending into the outer two-thirds of the dentate molecular layer (class II cells). This fraction dropped to 42% in 9-month-old animals (*** $p < 0.001$ vs. 3 months) and returned to slightly above 50% in middle-aged mice (53%: *** $p < 0.001$, vs. 3 months). (P) Age-dependent changes in total "BrdU+" and "BrdU−" DCX+ cells. DCX+ cells which incorporated BrdU (BrdU+/DCX+) dropped approximately 70% every 3 months (69.3% at 6 months, *** $p < 0.001$; 76.9% from 6 to 9 months, $$$ $p < 0.001$; 72.8% from 9 to 12 months, $+p = 0.037$), declining by 98.1% between 3 and 12 months. Total DCX+ cells which did not incorporate BrdU (BrdU−/DCX+) also declined with age (-70.6% at 12 months) but comparatively less than BrdU+/DCX+ cells (28.1% at 6 months; 34.7% from 6 to 9 months; 37.3% from 9 to 12 months). This differential decrement is illustrated by the BrdU−/BrdU+ DCX-expressing cell ratio which increases from a value of 0.58 at 3 months to a value of 6 at 12 months. (Q) The differential decline of BrdU+ and DCX+ cells. On average, DCX+ cells in the DG dropped 10.6% faster (** $p = 0.003$) than BrdU+ cells between 3 and 6 months (SGZ = -7.1%; GCL = -29.8%, +++), 14.9% faster (* $p = 0.016$) between 6 and 9 months (SGZ = -11%; GCL = -33.9%, +++), and 23.9% faster (** $p = 0.004$) between 9 and 12 months (SGZ = -15.8%; $; GCL = -62.6%, +++). These data confirm that DCX+ cells decrease relatively faster than BrdU+ cells during adulthood. Abbreviations: BrdU, 5-bromodeoxyuridine; DCX, doublecortin; DG, dentate gyrus; GCL, granule cell layer; SGZ, subgranular zone.
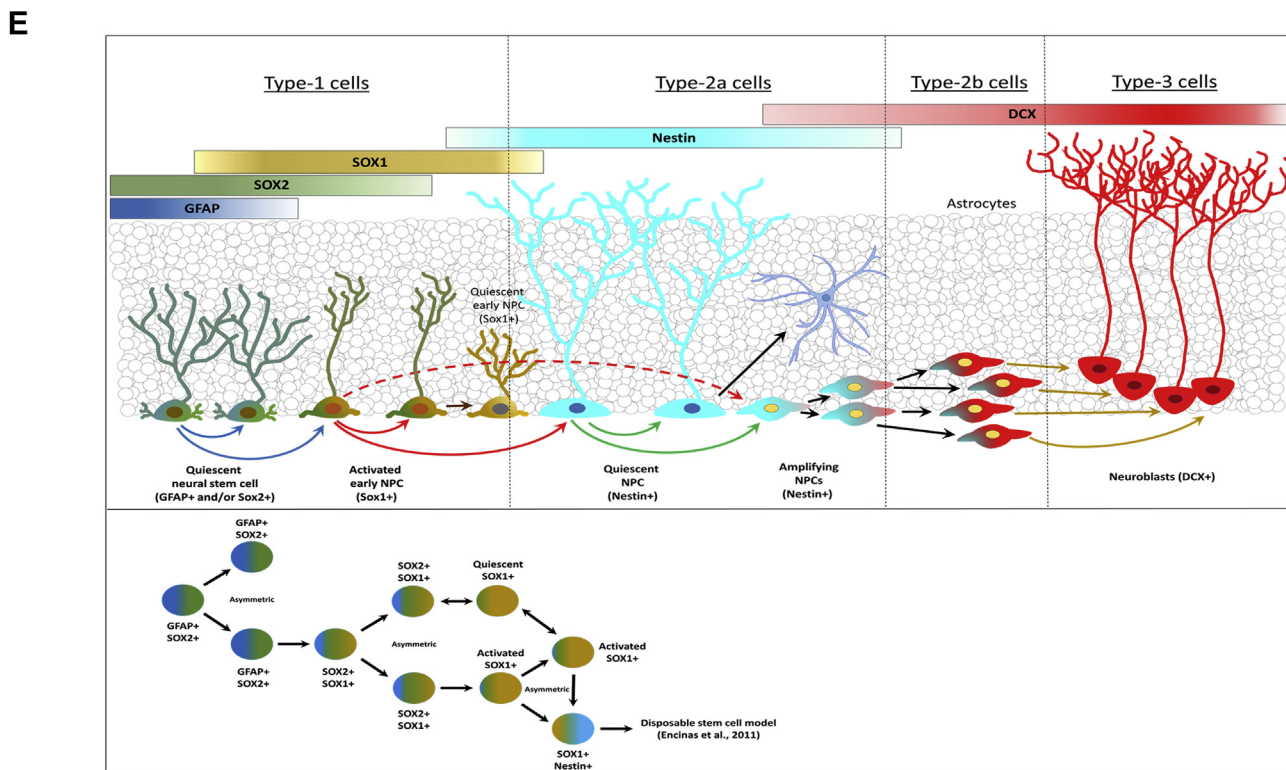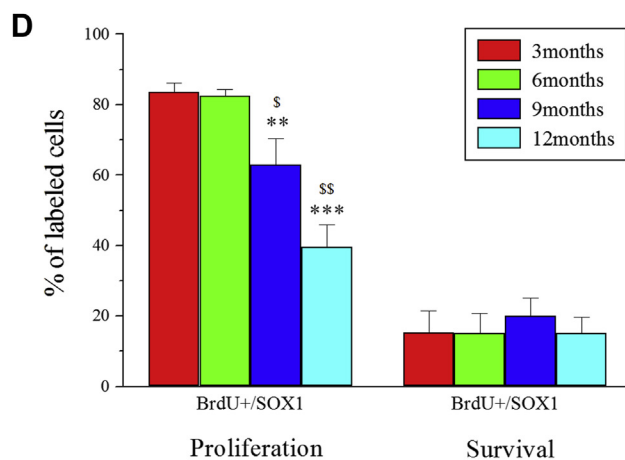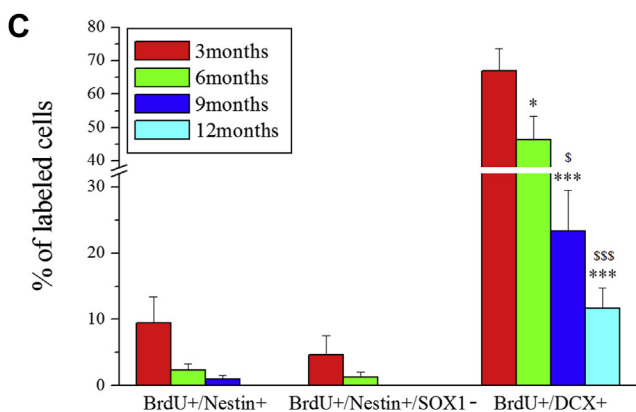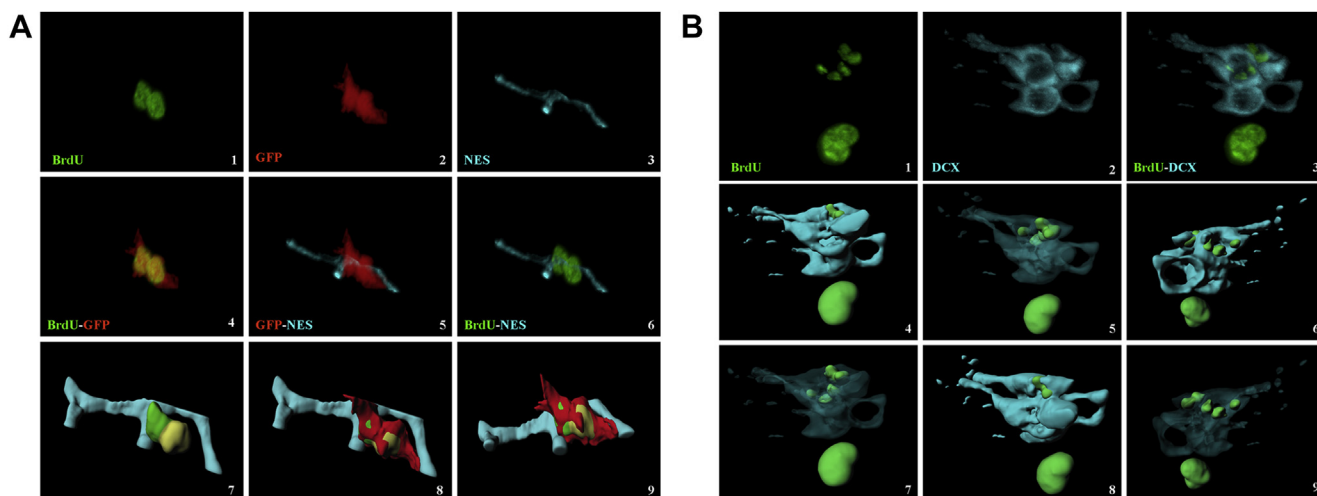
but not SGZ ($p > 0.05$). This was particularly evident in the 12-month-old DG which showed increased percentages of surviving BrdU+ cells compared with 3- ($p = 0.0027$), 6- ($p = 0.00035$), and 9-month-old animals ($p = 0.0079$) (Fig. 2I). Surviving 12-month-old animals also revealed significantly increased percentages of surviving GCL BrdU+ cells compared with 6 ($p = 0.0015$) and 9 month olds ($p = 0.003$) (Fig. 2I), illustrating a remarkable 2.5-time increase (+257%) in the 2-week survival rate. Collectively, the results demonstrate an age-dependent "relative" increase of surviving BrdU+ cells in the GCL, an effect particularly evident in 12-month-old animals.

3.3. Total DCX+ cells

Doublecortin is a microtubule-associated phosphoprotein, which regulates neuronal migration during development. In adult hippocampal neurogenesis DCX marks a period characterized by neurite and axon elongation between the committed progenitor cell stage (type-2 b/3) and the early postmitotic maturation stage, approximately 4–14 days after cell birth (Brown et al., 2003; Francis et al., 1999; Gleeson et al., 1999; Rao and Shetty, 2004). DCX+ cells in the DG receive synaptic GABAergic input and migrate into the inner third of the GCL. Here, dentate DCX expression was used as surrogate marker of neurogenesis (Couillard-Despres et al., 2005), to examine the impact of increasing age on total numbers of late progenitor cells (neuroblasts) and immature postmitotic newborn neurons. It should be mentioned however that DCX expression is not limited to the "canonical" neurogenic regions (hippocampus and SVZ/olfactory bulb) as DCX+ cells have been described in the adult striatum, corpus callosum, piriform cortex, amygdala, and hypothalamus (Batailler et al., 2014; Klempin et al., 2011; Marlatt et al., 2011; Martí-Mengual et al., 2013; Saaltink et al., 2012; Zhang et al., 2009).

In all age groups, DCX+ cell bodies were primarily located in the SGZ or inner third layer of the GCL (Fig. 3A–L). Aging significantly decreased total DCX+ in the SGZ ($p < 0.001$) (Fig. 3M), with the strongest reduction between 3 and 9 months. DCX+ cells decreased by 55.7% ($p < 0.001$), 54.5% ($p < 0.001$), and 44.2% ($p = 0.037$) between respectively 3–6, 6–9, and 9–12 months of age. A similar drop occurred in the GCL ($p < 0.001$) (Fig. 3M) although here the strongest loss of 63.6% ($p < 0.001$) occurred between 3 and 6 months followed by 50.3% ($p = 0.048$) and 40% between 6–9 and 9–12 months ($p > 0.05$). Together, aging significantly reduced DCX expression ($p < 0.001$) (Fig. 3M), with specific drops of 56.8% by 6 months ($p < 0.001$), 80.1% by 9 months ($p < 0.001$), and 88.8% by 12 months of age ($p < 0.001$) (Fig. 3N), constituting 56.7% during early adulthood ($p < 0.001$), 54% in mid-adulthood ($p < 0.001$), and 43.7% in middle age ($p = 0.047$) (Table 1).

Age-related influence on the fraction of younger (class I) and older (class II) DCX+ cells was also examined. Increasing age significantly reduced the percentage of class II cells (and, consequently, increased the fraction of class I cells) ($p < 0.001$). Class II cells declined from 71% to 62.6% and 41.9% from 3 to 6–9 months and increased to 52.5% at 12 months (Fig. 3O). This signifies a 12% reduction in the fraction of class II DCX+ cells at 6 months ($p = 0.068$), a 33% decline between 6 and 9 months ($p < 0.001$), and a 25.6% increase from 9 to 12 months ($p = 0.024$; nonsignificant when corrected for multiple comparisons). The fraction of class II cells was therefore reduced by 12% ($p = 0.068$), 41% ($p > 0.001$), and 26% ($p < 0.001$) by 6, 9, and 12 months, respectively (Fig. 3O).

As mentioned previously, DG DCX expression identifies the neuroblasts and/or immature neurons. We thus determined, for each animal, the size of the DCX cell population derived from NPCs which had proliferated (and, consequently, incorporated BrdU) 2 weeks before (total BrdU+/DCX+ cells) and those which had not (total BrdU−/DCX+ cells). Values were obtained by multiplying percentages of double-labeled BrdU+/DCX+ cells determined by confocal analysis (survival experiment) with the total numbers of DCX-labeled cells. Results showed that total BrdU+/DCX+ cells significantly declined with age ($p < 0.001$) (Fig. 3P). DCX+ cells derived from SGZ NPCs which had incorporated BrdU 2 weeks prior were reduced by 69.3% at 6 months ($p < 0.001$), 92.9% at 9 months ($p < 0.001$), and 98.1% at 12 months of age ($p < 0.001$), coinciding with a 69.3% decrease during early adulthood ($p < 0.001$), 76.9% during mid-adulthood ($p < 0.001$), and 72.8% up to middle age ($p = 0.037$). Likewise, total BrdU−/DCX+ cells also declined with age ($p = 0.003$) but at a lower rate (Fig. 3P). BrdU−/DCX+ cells dropped by 28.1% ($p > 0.05$), 53.1% ($p = 0.005$), and 70.6% ($p < 0.001$) at 6, 9, and 12 months, reflecting a drop of 28.1% during early adulthood ($p > 0.05$), 34.7% during mid-adulthood ($p > 0.05$), and a 37.3% up to middle age ($p > 0.05$). This differential decrement is illustrated by the BrdU+/BrdU ratio (Fig. 3P). If 3-month-old mice had approximately 2 BrdU+/DCX+ cells for each BrdU−/DCX+ cell (ratio = 0.58), this ratio increased to 1.5-to-1 at 6 months (ratio = 1.39), 2.5-to-1 at 9 months (ratio = 2.5), and 6-to-1 at 12 months (ratio = 6). Together, this illustrates a substantial age-dependent decline in total number of neuroblasts and/or immature neurons, occurring primarily during early and mid-adulthood. Interestingly, this reduction seems to be because of reduced proliferation and, to a lesser extent, total loss of NPCs.

3.4. Differential age-dependent decline of BrdU+ and DCX+ cells

Aging reduced both DG BrdU+ and DCX+ cells although DCX+ declined faster than BrdU+ in all age groups. Analysis of percentage

Fig. 4. Phenotype of DG newborn cells. To determine age-related effects on the phenotype of proliferating cells, immunofluorescent stainings were performed to investigate coexpression of BrdU with Nestin, Sox1, and DCX. (A) Representative confocal images ($A_{1–6}$) and 3-dimensional reconstructions ($A_{7–9}$; created with IMARIS, Bitplane) of DG cells positive for BrdU (green), Sox1 (red) and Nestin (light blue). (B) Confocal images ($B_{1–3}$) and 3-dimensional reconstructions ($B_{4–9}$) of double BrdU- (green) and DCX-labeled cells (light blue) in the GCL. (C and D) Age-dependent reduction in the percentage of BrdU+/Nestin+, BrdU+/Nestin/Sox1−, BrdU+/DCX+ and BrdU+/Sox1+ cells. Percentages of BrdU+/Nestin+ cells (regardless of Sox1 co-labeling) declined from 9.5% at 3 months to 2.3% at 6 months, and 1% at 9 months. By 12 months, BrdU+/Nestin+ cells were no longer detected (C). In the Sox1-negative fraction, the percentage dropped from 4.8% at 3 months to 1.1% and 0.5% at 6 and 9 months. Percentages of BrdU+/DCX+ cells gradually decreased from 66.8% at 3 months to 46.2% at 6 (* $p = 0.027$), 23.3% between 6 and 9 months (*** $p < 0.001$ vs. 3 months; \$ $p = 0.016$ vs. 6 months) and 11.7% between 9 and 12 months (*** $p < 0.001$ vs. 3 months; \$\$\$ $p < 0.001$ vs. 6 months; $p > 0.05$ vs. 9 months). Percentages of early NPCs which had just divided (BrdU+/Sox1+; proliferation experiment) remained stable between 3 and 6 months (83.6% and 82.5%, respectively), but sharply declined to 62.8% at 9 months (** $p = 0.009$ vs. 3 months; \$ $p = 0.012$ vs. 6 months) and 39.6% at 12 months (*** $p < 0.001$ vs. 3 and 6 months; \$\$ $p = 0.006$ vs. 9 months) (D, proliferation). Interestingly, percentages of BrdU+/Sox1+ in mice sacrificed 2 weeks after BrdU did not change with age, remaining 15%–18% from 3 to 12 months (D, survival). (E) A model of adult hippocampal neurogenesis. Adult NSCs/NPCs (type-1 cells) are located at the SGZ of the DG. Two different types of such cells are present, type 1 (GFAP+ and/or Sox2+) and type 1/2a (Sox2+, Sox1+, and/or Nestin+), which possibly maintain a reciprocal lineage relationship in the hippocampal niche. Type 1 cells represent rarely dividing radial glia-like stem cells, whereas type 1/2a cells represent rapidly and transiently proliferating NSCs/NPCs. Multipotent GFAP+/Sox2+ radial glia stem cells give rise (through asymmetric divisions) to multipotent Sox1+ neuroprogenitors which include 2 distinct subpopulations: activated (rapidly and transiently proliferating) and quiescent Sox1+ cells. It is intriguing to speculate that quiescent Sox1+ neuroprogenitors represent a reservoir of early NPCs capable of reactivation (and possibly differentiation into Nestin+ cells) in response to appropriate stimuli, ensuring a limited but constant source of new neurons into senescence therefore attenuating age-related loss of neuronal committed Nestin+ cells. In contrast, activated Sox1 cells divide asymmetrically to give rise to neural committed Sox1+/Nestin+ neuroprogenitors which further divide, differentiate, and develop into mature neurons (that will eventually integrate into the granular zone of the DG) and astrocytes, as illustrated by Encinas et al. (2011) in their "disposable stem cell model". Abbreviations: DG, dentate gyrus; GCL, granule cell layer; NPCs, neuroprogenitor cells; NSCs, neural stem cells; SGZ, subgranular zone. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
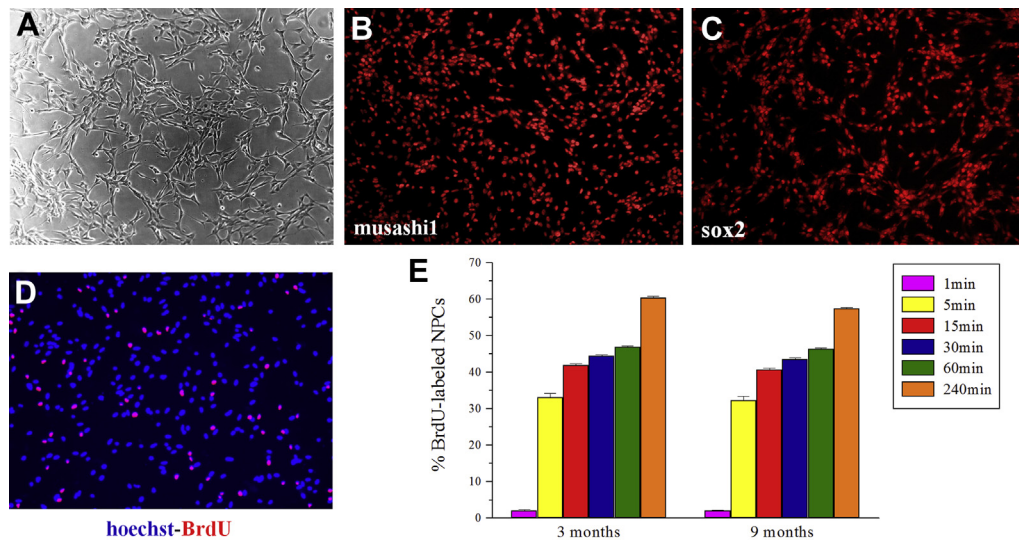
Fig. 5. Characterization and in vitro proliferation of 3- and 9-month-old hippocampal NPCs. NPCs were isolated from the hippocampus of 3- and 9-month-old mice. (A) NPCs grown in self-renewing conditions in the presence of growth factors (EGF and FGF). Most cells were positive for the neural progenitor specific RNA-binding protein Musashi1 (B) and the transcription factor Sox2 (C). (D) Proliferating NPCs which incorporated the thymidine analog BrdU. Proliferative rates were investigated by adding BrdU to the medium for different intervals (1, 5, 15, 30, 60, and 240 minutes) and, subsequently, measuring the percentages of BrdU-labeled cells. As depicted in E, the percentage of labeled cells increased with the duration of BrdU exposure from approximately 2% after 1 minute to approximately 60% after 4 hours. No differences were observed however in the rates of proliferation between 3- and 9-month-old NPCs. Abbreviations: BrdU, 5-bromodeoxyuridine; NPCs, neuroprogenitor cells.

change of DCX+ and BrdU+ cells from the survival experiment revealed a decline of DG DCX+ cells of 56.8% at 6 months (SGZ = −55.7%; GCL = −63.6%), 80.1% at 9 months (SGZ = −79.8%; GCL = −81.9%), and 88.8% at 12 months (SGZ = −88.7%; GCL = −89.1%) (Fig. 3Q). BrdU+ decreased 45.1% (SGZ = −47.2%; GCL = −34.9%), 64.9% (SGZ = −68.4%; GCL = −47.5%), and 65.1% (SGZ = −73.2%; GCL = −25.9%) at 6, 9, and 12 months (Figs. 2B and 3Q). On average, the reduction in DCX+ cells was thus 10.6% (p = 0.003) greater than BrdU during early adulthood (SGZ = −7.1%, p > 0.05; GCL = −29.8%, p < 0.001), 14.9% (p = 0.016) greater during mid-adulthood (SGZ = −11%, p > 0.05; GCL = −33.9%, p = 0.001),

and 23.9% (p = 0.004) greater during late adulthood (SGZ = −15.8%, p = 0.049; GCL = −62.6%, p = 0.002). These data confirm that DCX+ cells decrease relatively faster than BrdU+ cells during adulthood as the percentage of DCX+ cells dropped about 50% every 3 months. In contrast, percentual reduction of BrdU+ cells was strongest during early adulthood, decreasing progressively thereafter.

3.5. Phenotype of hippocampal proliferating cells

To determine age-related effects on the phenotype of DG proliferating cells, we investigated coexpression of BrdU with specific

Table 1
Summary of age-related changes of hippocampal neurogenesis

Readout	Early adulthood (%)	Mid-adulthood (%)	Middle age (%)	Age-related effect (3–12 mo) (%, p-value)
Total Sox1				
SGZ	NS	NS	NS	NS
Total BrdU: proliferation				
SGZ	−37.8	−54.6	NS	<0.001
GCL	NS	NS	NS	NS
DG	−33.4	−51.7	NS	−78.7, <0.001
Total BrdU: survival				
SGZ	−47.2	−40.2	NS	<0.001
GCL	NS	NS	NS	NS
DG	−45.1	−36	NS	−65.1, <0.001
Total DCX				
SGZ	−55.7	−54.5	−44.2	<0.001
GCL	−63.6	−50.3	NS	<0.001
DG	−56.7	−54	−43.7	−88.8, <0.001
Total BrdU+/DCX+				
DG	−69.3	−76.9	−72.8	−98.1, <0.001
Total BrdU−/DCX+				
DG	NS	NS	NS	−70.6, 0.003
% BrdU+/Nestin+				
DG	NS	NS	—	0.03
% BrdU+/SOX1+: proliferation				
DG	NS	−23.8	−37	<0.001
% BrdU+/DCX+: survival				
DG	−30.8	−49.5	−50	<0.001

Key: BrdU, 5-bromodeoxyuridine; DCX, doublecortin; DG, dentate gyrus; GCL, granule cell layer; NS, not significant; SGZ, subgranular zone.

neural antigens such as Nestin (marker of putative NPCs), Sox1 (marker of early NPCs), and DCX (marker of neuroblasts/immature granule neurons) (Kempermann et al., 2004; Zhao et al., 2008). Percentages of BrdU+/Nestin+/Sox1+ cells were determined using hippocampal slices from animals sacrificed immediately after BrdU administration (proliferation experiment) (Fig. 4A and C). Percentages of BrdU+/Sox1+/DCX+ cells were obtained from animals sacrificed 2 weeks after BrdU (survival experiment) (Fig. 4B and C).

We observed a significant age-related effect on the percentage of proliferating Nestin+ ($p = 0.03$) and DCX+ cells ($p < 0.001$) (Fig. 4C). A significant age-dependent reduction was also found in the percentage of BrdU+/Sox1+ cells from the proliferation ($p < 0.001$) (Fig. 4D) but not survival experiment ($p > 0.05$) (Fig. 4D). Post-hoc analysis revealed that the percentage of BrdU+/Nestin+ cells (regardless of Sox1 colabeling) declined from 9.5% at 3 months to 2.3% at 6 months and 1% at 9 months. By 12 months, BrdU+/Nestin+ cells were no longer detected (Fig. 4C). In the Sox1-negative fraction, the percentage dropped from 4.8% at 3 months to 1.1% and 0.5% at 6 and 9 months (Fig. 4C). In contrast, the percentage of early NPCs which had just divided (BrdU+/Sox1+; proliferation experiment) remained stable between 3 and 6 months of age (83.6% and 82.5%, respectively) but sharply declined to 62.8% at 9 months ($p = 0.009$ vs. 3 months; $p = 0.012$ vs. 6 months) and 39.6% at 12 months ($p < 0.001$ vs. 3 and 6 months; $p < 0.006$ vs. 9 months) (Fig. 4D; proliferation). Interestingly, percentages of BrdU+/Sox1+ in mice sacrificed 2 weeks after BrdU did not change with age, remaining 15%—18% from 3 to 12 months (Fig. 4D; survival). Finally, percentages of BrdU+/DCX+ cells gradually decreased from 66.8% in 3-month-old mice to 46.2% ($p = 0.027$ vs. 3 months), 23.3% ($p < 0.001$ vs. 3 months; $p = 0.016$ vs. 6 months), and 11.7% ($p < 0.001$ vs. 3 months; $p < 0.001$ vs. 6 months; $p > 0.05$ vs. 9 months) in 6-, 9-, and 12-month-old mice (Fig. 4C) (Table 1).

3.6. Age-dependent effects on NPC proliferation: in vitro approach

Shrinkage of the hippocampal NPC pool has recently been proposed as the main force driving age-related neurogenesis decline (Encinas et al., 2011), which contradicts beliefs that pool size remains constant although NPCs proliferative capacity progressively diminishes. To examine age-mediated influences on proliferative rates, NPCs were isolated from hippocampi of 3- and 9-month-old mice, cultured in self-renewing conditions in the presence of growth factors (EGF and FGF) (Fig. 5A) and characterized by immunocytochemistry (Fig. 5B and C). Proliferating cells were mostly positive for the neural progenitor specific RNA-binding protein Musashi1 (Fig. 5B) and the transcription factor Sox2 (>98%; Fig. 5C). Proliferation rates were investigated by exposing NPCs to BrdU for different intervals (1, 5, 15, 30, 60, and 240 minutes) followed by determination of BrdU incorporation (Fig. 5D). The percentage of labeled cells increased with the duration of BrdU exposure from approximately 2% after 1 minute to approximately 60% after 4 hours ($p < 0.001$) (Fig. 5E). No differences were observed however in proliferation level between NPCs isolated from 3- and 9-month-old brains ($p > 0.05$), suggesting that declining age-related hippocampal neurogenesis does not result from reduced responsiveness of aging NPCs to normal growth factor-mediated signaling. These results should however be interpreted with caution as in vitro conditions have been shown to differentially affect the proliferation of different classes of NPCs (Doetsch et al., 2002). For instance, exogenous EGF has been shown to strongly stimulate the proliferation of rapidly transit-amplifying cells while having limited effects on primary and less proliferative stem cells. This was also confirmed here as NPCs cultures were highly homogenous and rich of Sox2+ NPCs (>98%). In vitro results might thus indicate that increasing age

from 3 to 9 months did not affect the responsiveness of a specific subpopulation of early NPCs (possibly early type-2 cells) to high EGF/FGF concentrations. Albeit intriguing to speculate, this may not provide a complete picture of how increasing age affects the diverse range of early and late NPCs which populate the hippocampal niche.

4. Discussion

Hippocampal neurogenesis occurs throughout life in various species, including humans (Altman and Das, 1965; Eriksson et al., 1998; Spalding et al., 2013) and steadily decreases during aging (Klempin and Kempermann, 2007; Knoth et al., 2010; Kuhn et al., 1996). Although well established, the mechanisms behind this age-related phenomenon are unclear. A recent study indicated depletion of Nestin+ NPCs as the main cause of age-associated neurogenesis decline, although it did not clarify whether additional mechanisms were involved (Encinas et al., 2011). The present study focused on changes in hippocampal neurogenesis occurring between 3 and 12 months of age. Distinctions were made between 3 periods: early (3—6 months), middle (6—9 months), and late adulthood (9—12 months). For each interval, changes in proliferating, differentiating, and surviving newborn cells were examined and summarized as follows (see Table 1):

- Total proliferating (BrdU+) cells decreased by 79%, with the strongest relative drop during mid-adulthood (-52%).
- Total 2-week-old BrdU+ cells declined by 65%, with the greatest relative reduction during early adulthood (-45%).
- Total numbers of DCX+ neuroblasts and immature neurons dropped by 89%, declining approximately 50% every 3 months.
- Total early Sox1+ NPCs declined by 30% between 3 and 12 months, a moderate although nonsignificant reduction.
- The percentage of proliferating (BrdU+) Sox1-expressing cells decreased by 53%, with the strongest relative reduction between 9 and 12 months (-37%).
- The percentage of proliferating (BrdU+) Nestin-expressing cells fell by 95% between 3 and 9 months. By 12 months, no BrdU+/Nestin+ cells were detected.
- The percentage of BrdU+/DCX+ cells declined by 83% between 3 and 12 months, with the strongest relative reduction during mid and late adulthood (-50%).
- No in vitro differences were found in the proliferation of NPCs isolated from 3- to and 9-month-old hippocampi.

4.1. Age-related influences on total proliferating and surviving cells (BrdU+)

We first examined how progressing age affected rates of cell proliferation and newborn cell survival using BrdU immunohistochemistry. Our results confirmed a dramatic reduction of total DG proliferation, with the strongest relative decrement during mid-adulthood (-55%). By 12 months, total proliferating cells dropped by 79% (Fig. 2A and B). As only a fraction of adult-born cells is reported to persist for longer periods (Dayer et al., 2003), we also examined 2-week-old BrdU+ cells and found a similar age-associated reduction in total newborn cell survival (Fig. 2A and B). With the strongest relative drop in early adulthood, this decrement leveled off by 9 months, reaching a final 65% reduction by 12 months. Based on the studied timeframe, these data suggest that increasing age up to middle age primarily affects hippocampal proliferation and, to a lesser extent, newborn cell survival, differing essentially in their temporal influences as proliferation is predominantly reduced between 6 and 9 months while cell survival slightly earlier (3—6 months).

Newborn cells arising from NPC proliferation migrate into the GCL where they differentiate into neurons and integrate into the local network (Cameron et al., 1993; Hastings and Gould, 1999; van Praag et al., 2002). This migration is reflected by the increased percentage of 2-week-old BrdU+ cells in the GCL (Fig. 2H). Although increasing age was associated with a relative increase of BrdU+ cell survival in the GCL, particularly around middle age, this is remarkable given observations of reduced SGZ-to-GCL migration of newborn cells in the aged brain (Heine et al., 2004). A similar spatial redistribution of BrdU+ cells within the DG as reported here has been observed in aged transgenic pNestin-GFP mice (Walter et al., 2011), suggesting that this phenomenon might be related to enhanced newborn cell survival during middle age. In accordance, although the "2-week survival rate" of BrdU+ cells in the DG fluctuated between 50% and 70% from 3 to 9 months, it rose to a staggering 100% at 12 months (Fig. 2I), due mostly to a 2.5-fold increase observed in the GCL. As neurogenesis is essential for proper DG morphogenesis and function, this structure seems to respond to perturbations that reduce proliferation and NSC/NPC numbers with compensatory responses to promote newborn cell survival (Ciaroni et al., 2002). In support of the latter, our results substantiate the view that age-related suppression of adult neurogenesis results from diminished production, and not reduced survival, of newborn cells which, in relative terms, appears notably enhanced in the middle-aged DG.

4.2. Age-related effects on total DCX+ cells

During adult hippocampal neurogenesis, DCX is expressed by neuroblasts and immature neurons during a period characterized by neurite and axonal elongation, approximately 4–14 days after cell birth (Brown et al., 2003). In this study, analysis of DG DCX expression allowed investigation of increasing age effects on total numbers of late NPCs (type-2 b/3) and immature newborn neurons. As for BrdU, a steady and robust decline was observed, between 3 and 12 months, in DCX+ cells (Fig. 3M and N). By 12 months, almost 90% of DCX+ cells seen at 3 months had disappeared, in line with previous reports (Cameron and KcKay, 1999; Heine et al., 2004; Kempermann et al., 1998; Kuhn et al., 1996). Notably, increasing age was also associated with a significant change in ratio between young class I cells (with no or short processes not extending beyond the molecular layer) and more mature class II cells (with at least 1 dendrite reaching into the molecular layer; Plumpe et al., 2006; Van Bokhoven et al., 2011). Although class II cells represented most of the DCX+ cells in 3- and 6-month-old animals, their fraction declined from 71% at 3 months to 63% at 6 months (Fig. 3O). This percentage further decreased to 42% at 9 months to return slightly above 50% at 12 months. These data indicate that aging not only reduces total numbers of DCX+ cells but also delays neuronal maturation as illustrated by increased percentages of young DCX+ cells (class I) and, consequently, reduced fractions of more mature, post-proliferative DCX+ cells (class II).

Further characterization of DCX+ cell population also revealed that, in contrast with previously findings (Rao et al., 2005, 2006; Walter et al., 2011), the fraction of DCX+ cells that incorporated BrdU gradually decreased from 67% at 3 months to 12% at 12 months of age (−83%; Fig. 4C). The reason for this discrepancy is unknown but might be related to the use of rats (Rao et al., 2005, 2006) instead of mice (present data) as granule cell maturation in mice has been shown to lag significantly behind that observed in rats (Snyder et al., 2009), a phenomenon which could be further amplified by age and thus explain the gradual reduction of the BrdU+/DCX+ cell fraction seen here between 3 and 12 months. Because Walter et al. (2011), examined age-related changes in BrdU+/DCX+ cells in mice sacrificed 2 hours following BrdU, it is

possible that the longer survival time used here (14 days) contributed to the differences between the 2 studies. Notably, total DCX+ cells incorporating BrdU underwent a 98% decline between 3 and 12 months, whereas the BrdU-negative fraction dropped "only" by 71% (Fig. 3P). This differential decline lead to a steady increase of the ratio between BrdU-negative and BrdU-positive DCX+ cells from approximately 0.5 at 3 moths to 6 at 12 months (Fig. 3P). Finally, comparison between total BrdU+ and DCX+ immunoreactivity revealed that DCX+ cells declined significantly faster than BrdU+ cells (−89% vs. −65%, respectively). Although evident in all age-groups (−11% in early adulthood and −15% in mid-adulthood), this differential decrement was especially apparent during late adulthood (−24%; Fig. 3Q) and might reflect an age-dependent change in fate-choice determination to induce a shift in NPC differentiation toward astrocytes as opposed to neurons. Importantly, astrocytic conversion has actually been proposed as a key mechanism behind age-related loss of NPCs (Encinas et al., 2011).

4.3. Age-related changes in total and proliferating Sox1+ NPCs

In their "disposable stem cell" theory, Encinas et al., (2011) showed that quiescent Nestin+ NPCs generate committed rapid amplifying NPCs by asymmetric cell division but, after only a few rounds of divisions, they differentiate into postmitotic astrocytes, depleting the DG NPC pool. We therefore explored age-related effects on the total size of earlier "progenitor" cells by quantifying changes in Sox1-expressing cell numbers within the hippocampal SGZ. Sox1 expression is critical in maintaining NPCs in a non-committed state (Bylund et al., 2003), marking an activated population of early NPCs that gives rise to most, if not all, newborn granular neurons (Venere et al., 2012). Notably, as Sox1 directly activates Nestin-enhancer elements, Sox1 expression precedes the appearance of Nestin (Kan et al., 2004; Tanaka et al., 2004). Remarkably, despite reports of severe age-related reduction of hippocampal Nestin+ NPC pool (Encinas et al., 2011), our results indicated that total numbers of earlier Sox1+ NPCs did not significantly change between 3 and 12 months (Fig. 1M). In line with our findings, a recent study investigating Sox2+ NPCs in 4-, 12-, and 24-month-old rats, also reported no difference between young and middle-aged animals with only a slight reduction during senescence (Hattiangady and Shetty, 2008). Although loss of early Sox1+ NPCs did not account for reduced neurogenesis up to 12 months of age, our results cannot exclude the possibility that this loss intensifies later in life to play a significant role after middle age.

It seems clear that increasing age up to middle age primarily reduces hippocampal neurogenesis by suppressing NSCs and/or NPCs proliferation. We then examined whether these suppressive influences equally affected all NPC subpopulations by comparing age-related changes in the fractions of proliferating Sox1+ and Nestin+ NPCs. Closer examination revealed that BrdU+/Sox1+ cells constitute approximately 80% of proliferating cells through early adulthood, dropping only during mid-adulthood (−23%) and middle age (−37%) (Fig. 4D). This initial fraction of proliferating Sox1+ cells lies above previous reports of 20% which is likely because of the inclusion of downstream neural subpopulations of committed progenitors, including Nestin+ NPCs (Encinas et al., 2011), as the time required for complete elimination of GFP, once Sox1 expression ceases and NPCs differentiate further, is unknown. This possibility seems confirmed by the percentage of BrdU+/Sox1+ cells detected in the survival experiment (approximately 15%; Fig. 4D) in which animals were sacrificed 14 days after BrdU administration, an interval sufficient to eliminate any residual and nonspecific GFP expression. The presence of a small but still consistent population of BrdU+/Sox1+ cells up to 2 weeks after BrdU administration also suggests that a fraction of activated Sox1+ NPCs may become

quiescent after 1 or more rounds of cell division (Fig. 4E). Alternatively, they could represent a different class of NPCs with repeated self-renewal and/or an unusually long G2/M phase. Remarkably, this percentage of 2-week-old BrdU+/Sox1+ cells did not change from early adulthood to middle age (Fig. 4D).

Nestin is considered an earlier marker of definitive neural commitment and is expressed by NPCs in the central nervous system from embryonic to adult stages (Kan et al., 2004; Tanaka et al., 2004). Here, a small population of low proliferating Nestin+ NPCs was detected at 3 months, accounting for 9.5% of the BrdU+ cells (Fig. 4C). This population dropped to 2.3% and 0.5% by 6 and 9 months. By 12 months, no double-labeled BrdU+/Nestin+ cells were found. Although proliferative rates seen here seem initially higher than those reported by Encinas et al. (2011), proliferating Nestin+ NPCs included both Sox1-positive (BrdU+/Sox1+/Nestin+) and Sox1-negative cells (BrdU+/Sox1−/Nestin+). Excluding the former, the percentage of "pure" BrdU+/Nestin+(Sox1−) cells dropped to 4.8%, 1.1%, and 0.5% at 3, 6 and 9 months, respectively (Fig. 4C). As multiple BrdU pulses were given, our results seem in range with previous reports.

4.4. Concluding remarks

Our findings indicate that the decline of hippocampal neurogenesis observed in mice between 3 and 12 months of age was mostly a result of reduced NSC/NPC proliferation. Adult hippocampal neurogenesis requires a specific molecular and cellular microenvironment that provides the signals to sustain and regulate proliferation of NPCs and differentiation of their progeny. Aging can affect neurogenic niche microenvironment, modifying blood-derived factors and local cues. Examination of NPCs isolated from 3- and 9-month-old hippocampi seems to confirm age-dependent changes in the local niche microenvironment, as no differences in proliferation were found, under self-renewing conditions, between young and older NPCs (Fig. 5E). These in vitro results should however be interpreted with caution as they may provide only a partial picture of the in vivo effects of aging on hippocampal neurogenesis. In vitro conditions have been shown to differentially affect NPC proliferation (Doetsch et al., 2002), enriching cell cultures of rapidly transit-amplifying cells (Sox2+; Fig. 5C) at the expense of less proliferative NPCs (GFAP+ and Nestin+). Our findings also show that increasing age targeted distinct subpopulations of hippocampal NPCs differently, with BrdU+/Sox1+ cells declining by only 53%, whereas BrdU+/Nestin+ and BrdU+/DCX+ cells dropping by 95% (already at 9 months) and 83%, respectively. Importantly, although total numbers of neuroblasts and immature neurons (DCX+ cells) steadily decreased with age, new glial cell production suddenly increased between 9 and 12 months of age. As a fraction of adult-born astrocytes derive directly from Nestin+ NPCs (Encinas et al., 2011), increased age-dependent rates of glial cell production may indicate that loss of such NPC subpopulation is not constant throughout adulthood but sharply accelerates around middle age. The almost complete disappearance of proliferating Nestin+ cells, the drastic reduction of DCX+ cells (particularly newborn BrdU+/DCX+ cells), and changes in fate-choice determination seem to support the "disposable stem cell" model put forth by Encinas et al. (2011). Our study however provides additional insight into the age-related mechanisms that regulate not only proliferation and differentiation but also the maintenance of the hippocampal NSC/NPC pool.

First, loss of committed Nestin+ NPCs, alone, cannot account for the striking age-dependent reduction of hippocampal neurogenesis during early- and mid-adulthood, especially in view of the limited change in numbers of earlier Sox1-expressing NPCs (Fig. 4E). Additional mechanisms must be implicated and our results point to increased quiescence of such early NPCs (and possibly NSCs) and/or a lengthened neuronal differentiation process. Notably, age-related delayed neuronal maturation as well as reduced type-1 and 2a cell proliferation have been previously observed (Hattiangady et al., 2008; Heine et al., 2004; Rao et al., 2005; Walter et al., 2011).

Second, survival experiments revealed the existence of a small population of NPCs which continue to express Sox1 (but not DCX+) 2 weeks after BrdU administration. It is intriguing to speculate that these 2-week-old BrdU+/Sox1+ cells could represent a reservoir of quiescent early NPCs capable of reactivation (and possibly differentiation into Nestin+ cells) in response to the appropriate sets of stimuli. These cells may therefore ensure a limited but constant source of new neurons into senescence, attenuating age-related loss of Nestin+ cells (Fig. 4E). These quiescent and not yet committed Sox1+ NPCs could represent a key target for interventions directed at enhancing neurogenesis during old age.

Finally, as declined neurogenesis rates may be linked to age-related changes in hippocampal neurogenic niche microenvironment, interventions should thus be directed toward protecting or reversing deterioration of hippocampal environment. Pharmacologic manipulation of neurotrophic factors, growth factors, hormones, or neurotransmitter levels represent plausible approaches to attenuate or delay age-related decline of neurogenesis. Environmental enrichment and voluntary exercise have emerged as promising and low-cost strategies to boost NPC proliferation. Physical activity is a robust stimulus for adult hippocampal neurogenesis in rodents from birth to the oldest age (Kannangara et al., 2011; Kronenberg et al., 2003; Marlatt et al., 2012; Steiner et al., 2008; van Praag et al., 2005, 1999; Wu et al., 2008). Running has the potential to limit the massive decrease in neurogenesis observed in aged animals (Kronenberg et al., 2006), an action which seems directly linked to the stimulation of NPC proliferation (Kronenberg et al., 2003; Lugert et al., 2010; Steiner et al., 2008) and/or shortening of cell cycle length (Farioli-Vecchioli et al., 2014). Housing rodents in enriched environments has also been shown to increase hippocampal neurogenesis, an effect maintained in old age (Kempermann et al., 1998). Notably, combination of exercise and environmental enrichment results in a greater increase in neurogenesis than either exercise or enrichment alone (Fabel et al., 2009; Olson et al., 2006). Most importantly, exercise and environmental enrichment (including mental training) are accessible to most individuals and could accomplish the same goal as pharmacologic treatments without additional side effects (Marlatt et al., 2010). Unfortunately, environmental influences were not investigated here. Although mice were provided with some environmental enrichment (nesting material and novel objects), they lacked the rich and diverse stimuli that characterize wild life and profoundly affect neurogenesis. Laboratory animals face very different environmental challenges than their free-living counterparts, and caution must be used when translating laboratory findings to a natural or wild population. This is particularly important when determining the consequences of experimental conditions (both positive and negative) on neurogenesis in the laboratory as these effects may differ considerably from a natural population. Despite these limitations, enriched environment- and exercise-induced boost of hippocampal neurogenesis may prove effective in alleviating or delaying those debilities associated with normal and pathologic aging.

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