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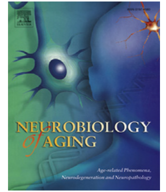

Changes in hippocampal neurogenesis throughout early development[☆]

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A B S T R A C T

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 (bright-light 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).

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