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***In vivo* contribution of nestin- and GLAST-lineage cells to adult hippocampal neurogenesis**

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Abstract

Radial glia-like cells (RGCs) are the hypothesized source of adult hippocampal neurogenesis. However, the current model of hippocampal neurogenesis does not fully incorporate the *in vivo* heterogeneity of RGCs. In order to better understand the contribution of different RGC subtypes to adult hippocampal neurogenesis, we employed widely-used transgenic lines (Nestin-CreER^{T2} and GLAST::CreER^{T2} mice) to explore how RGCs contribute to neurogenesis under basal conditions and after stimulation and depletion of neural progenitor cells. We first used these inducible fate-tracking transgenic lines to define the similarities and differences in the contribution of nestin- and GLAST-lineage cells to basal long-term hippocampal neurogenesis. We then explored the ability of nestin- and GLAST-lineage RGCs to contribute to neurogenesis, after experimental manipulations that either ablate neurogenesis (i.c.v. application of the anti-mitotic AraC, cytosine-β-D-arabino-furanoside) or stimulate neurogenesis (wheel running). Interestingly, in both ablation and stimulation experiments, labeled RGCs in GLAST::CreER^{T2} mice appear to contribute to neurogenesis, whereas RGCs in Nestin-CreER^{T2} mice do not. Finally, using NestinGFP reporter mice, we expanded on previous research by showing that not all RGCs in the adult dentate gyrus subgranular zone express nestin, and therefore RGCs are antigenically heterogeneous. These findings are important for the field, as they allow appropriately conservative interpretation of existing and future data that emerge from these inducible transgenic lines. These findings also raise important questions about the differences between transgenic driver lines, the heterogeneity of RGCs, and the potential differences in progenitor cell behavior between transgenic lines. As these findings highlight the possible differences in the contribution of nestin and GLAST lineage cells to long-term neurogenesis *in vivo*, they indicate that the current models of hippocampal neurogenesis should be modified to include RGC lineage heterogeneity.

Keywords

subgranular zone; dentate gyrus; genetic fate tracking; radial glial cell; Cre recombinase

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Introduction

The putative stem cells of the hippocampal subgranular zone (SGZ) are radial glia-like cells (RGCs; also called Type-1 cells (Kempermann et al., 2004), Type-B cells (Seri et al., 2001), or quiescent neural precursors (Encinas et al., 2011)). Models of adult hippocampal neurogenesis typically depict RGCs dividing asymmetrically to produce progenitors, some of which survive and give rise to dentate gyrus granule cell (GC) neurons (Seri et al., 2001; Kempermann et al., 2004; Lagace et al., 2007; Encinas et al., 2011). This model has been established by using multiple techniques. The first study to characterize RGCs as the putative stem cells ablated dividing neural precursor cells *in vivo* by infusing the anti-mitotic drug cytosine- β -D-arabinofuranoside (AraC) (Seri et al., 2001). Via histology and electron microscopy, these authors identified the first cells to divide after ablation as cells with radial glial morphology and astrocytic properties, and postulated that these glial cells are the stem cells in the hippocampus. The authors followed up these initial results using selective viral transduction strategies and found that cells expressing nestin or GFAP give rise to adult-born hippocampal neurons (Seri et al., 2004). Around the same time, two different groups characterized the electrophysiological properties of RGCs from NestinGFP mice (Filippov et al., 2003; Fukuda et al., 2003), which provided additional evidence that RGCs had properties of astrocytes. Based on these studies, it was presumed that the RGC was the stem cell that supported adult neurogenesis (Kempermann et al., 2004), and this model is widely referred to in the literature.

While the current model is highly useful, it falls short in regards to clarifying the role of RGCs in the process of neurogenesis in three key ways. First, the model assumes a single type of RGC exists, and that RGCs uniformly express the same markers. This is in spite of recent data suggesting that SGZ RGCs comprise antigenically heterogeneous subpopulations (Kempermann et al., 2004; Steiner et al., 2006; Kim et al., 2007; Seki et al., 2007), and the existence of subpopulations of stem cells that express different markers during early life cortical neurogenesis. Differential expression of markers has functional importance, as subpopulations during embryonic neurogenesis may give rise to different populations of neurons (Hartfuss et al., 2001; Liang et al., 2012). In the subventricular zone – the other well-accepted region of adult neurogenesis – there may also be location-specific subpopulations of stem-like cells that produce daughter cells with different fates (Merkle et al., 2007; Platel et al., 2009). Second, the model asserts that all RGCs maintain a capacity to divide and contribute to neurogenesis. The functional importance of RGC subpopulations *in vivo* remains unexplored, and correlative studies with reporter mice do not clarify which RGC subpopulations produce the neurogenic progenitors that ultimately produce neurons (Suh et al., 2007; Lugert et al., 2010). Third, the model asserts that RGCs give rise to neurons, but it is unclear whether RGCs maintain multi-lineage potential *in vivo* (Lagace et al., 2007; Bonaguidi et al., 2011; Dranovsky et al., 2011; Encinas et al., 2011; Bonaguidi et al., 2012). Clarification of the *in vivo* contribution of RGC subtypes to adult hippocampal neurogenesis is a challenging but critical step in advancing our understanding of stem cells in the adult brain and the process of adult hippocampal neurogenesis.

In order to further clarify which cells give rise to adult hippocampal neurons *in vivo* and to identify whether there are antigenically heterogeneous RGCs, we utilized three different transgenic mouse lines. Two inducible transgenic mouse lines (Nestin-CreERT²/R26R:YFP and GLAST::CreERT²/R26R:YFP) were used for comparative fate-tracking of nestin and GLAST lineage cells during basal neurogenesis, as well as after perturbations of neurogenesis (chemical-induced ablation and running-induced stimulation of proliferative cells). A third reporter mouse line (NestinGFP) was also used to characterize whether RGC subpopulations undergo mitosis. Our data highlight differences between mouse lines,

suggest *in vivo* heterogeneity of RGCs, and show the differential *in vivo* contribution of nestin and GLAST lineage cells to adult hippocampal neurogenesis.

Methods

Animals

Mice were housed on a 12-hour (h) light/dark cycle in a pathogen-free facility at UT Southwestern approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Procedures and husbandry were in accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals, approved by the UT Southwestern Medical Center Animal Care and Use Committee, and designed to minimize animal number, pain, and suffering. Homozygous Nestin-CreERT² transgenic mice [(Lagace et al., 2007); Jackson Laboratories stock #: 016261] on a C57BL/6 background were crossed to homozygous Rosa26Reporter:YFP (R26R:YFP, Jackson Laboratories stock #:006148) knock-in mice to produce Nestin-CreERT²/R26R:YFP (“Nestin-Cre/YFP”) bitransgenic mice hemizygous for both genes. Genotyping and characterization for the Nestin-Cre/YFP line are described elsewhere (Battiste et al., 2007; Lagace et al., 2007; Ables et al., 2010). Our current work extends previously published work (Lagace et al., 2007); for quantification and phenotypic analyses, Nestin-CreERT²/R26R:YFP mice were born within a 6-month window in 2006, administered Tamoxifen (TAM; below) and collected at various time points. Previously published results are indicated in the figures and within the text; these data are presented here for comparison to other time points and other mouse lines in this study (Fig.1d–f). Homozygous GLAST::CreERT² knock-in mice on a mixed C57/S129 background were crossed with R26R:YFP knock-in mice on a C57BL/6 background to produce GLAST::CreERT²/R26R:YFP (“GLAST::Cre/YFP”) bitransgenic mice heterozygous for GLAST::CreERT² and hemizygous for R26R:YFP. Genotyping and characterization for the GLAST::Cre/YFP line are described elsewhere (Ninkovic et al., 2007). To control for background and gene-dosage effects, only GLAST::Cre/YFP mice of the third intercross were used. Homozygous NestinGFP transgenic mice (Yamaguchi et al., 2000) were bred on a C56BL/6 background.

Recombination in CreERT² mice

To induce recombination in Cre/YFP lines, young adult mice (5–6 weeks) of both sexes received TAM (180 mg/kg/day i.p., 5 days [d]) at 30 mg/ml (in 10% EtOH in sunflower seed oil) (Lagace et al., 2007). TAM, EtOH, and oil were purchased from Sigma.

Surgery and intracerebroventricular infusion (i.c.v)

25d post-TAM, Nestin-Cre/YFP and GLAST::Cre/YFP mice were surgically implanted unilaterally with i.c.v. cannulae (PlasticsOne) attached to osmotic minipumps (flow 0.5 µl/h, 7d, Alzet) that delivered either cytosine-β-D-arabinofuranoside hydrochloride (AraC, 2% w/v, Sigma) or saline (Seri et al., 2001; Mak et al., 2007). Targeting coordinates from bregma were A/V +0.2mm, ML –0.8mm, and DV –2.3mm.

Running (voluntary physical activity)

~125d post-TAM, Nestin-Cre/YFP and GLAST::Cre/YFP mice were singly-housed with a running wheel (Coulbourn Instruments) that was either locked (unable to turn; control group) or open (running group) (Ables et al., 2010). Mice had free access to wheels for ~14d before sacrifice.

BrdU administration

To assess proliferation of RGCs in NestinGFP mice, 10-week-old male and female mice were given 4 injections of BrdU (Boehringer Mannheim, 150 mg/kg, 10 mg/ml, i.p. 1inj/6h). Mice were perfused 2h after the last injection. To assess proliferation and neurogenesis in Nestin-Cre/YFP and GLAST::Cre/YFP mice, mice were given a single BrdU (150 mg/kg) at various times post-TAM and brains were collected 2h later (Lagace et al., 2007; Ables et al., 2010).

Tissue collection and preparation

Mice were anesthetized and perfused intracardially with 0.1M PBS followed by perfusion in 4% paraformaldehyde in 0.1M PBS (Lagace et al., 2007; Ables et al., 2010). Extracted brains were post-fixed overnight in 4% paraformaldehyde followed by cryopreservation in 30% sucrose with 0.1% sodium azide in 0.1M PBS. Sections (30 μ m) throughout the entire hippocampus were cut on freezing microtome in 1:4 (RGC proliferation) or 1:9 series (remaining analyses) and stored free floating in 0.1% sodium azide in 0.1M PBS at 4°C until stained.

Antibodies

Primary antibodies used for slide-mounted (SM) or free-floating (FF) immunohistochemistry (IHC) were as follows: rabbit anti-brain lipid binding protein (BLBP, Abcam, 1:500FF); rat anti-bromodeoxyuridine (BrdU, Accurate, 1:400SM, full-pretreatment); goat anti-doublecortin (DCX, Santa Cruz, 1:100-1:500FF); rabbit anti-glial fibrillary acidic protein (GFAP, DAKO, 1:3000FF); mouse anti-GFAP (Millipore, 1:2000-1:3000FF); guinea pig anti-glutamate aspartate transporter (GLAST, Millipore, 1:6000FF); mouse anti-nestin (BD Pharmingen, 1:1000FF); mouse anti-NeuN (Millipore, 1:800FF); rabbit anti-S100 β (Swant, 1:10,000FF); goat anti-Sox2 (Santa Cruz, 1:800-1:1000FF); rabbit anti-Sox2 (Millipore, 1:3000FF); rabbit anti-Tbr2 (Abcam, 1:200FF). For nestinGFP detection, we used rabbit anti-GFP (Invitrogen, 1:10,000FF) and chicken anti-GFP (Aves, 1:10,000FF). For Rosa:YFP detection for phenotypic analysis, we used rabbit anti-GFP or chicken anti-GFP at 1:1000FF; for Rosa:YFP detection for quantification, we used rabbit anti-GFP at 1:3000SM with citric acid pretreatment, plus tyramide signal amplification (TSA).

Immunohistochemistry

IHC was performed essentially as described (Lagace et al., 2007; Ables et al., 2010). Sections were coded during IHC to blind the observer to experimental condition and mouse line; the code was not broken until all analyses were complete. For SM IHC, sections were mounted onto charged glass slides before staining; for FF IHC, sections were stained in 24-well plates. For citric acid pretreatment, SM sections were incubated in 0.01M citric acid (pH 6.0) for 15 min at 100°C. Comprehensive detection of BrdU required citric acid pretreatment, followed by 10 min permeabilization (0.1% trypsin in 0.1% CaCl₂/0.1M Tris-HCl) followed by DNA denaturation for 30 min (2M HCl). For SM and FF IHC, nonspecific binding was blocked with 3% donkey serum (EquiTech) and 0.3% Triton X-100 in 1xPBS for 30–60 min at room temperature. Primary antibodies were diluted in 3% donkey serum and 0.3% Tween-20 in 1xPBS, and sections were incubated overnight at room temperature. Detection of GLAST, nestin, and S100 β required 2–3 night incubation in primary at 4°C. Secondary antibodies (1:500FF, 1:200SM; Jackson ImmunoResearch) were diluted in 1.5% donkey serum in 1xPBS. Most antibodies were detected with fluorophore-conjugated secondaries. Signals for rat anti-BrdU and rabbit anti-GFP for quantitative analysis of YFP+ cells were amplified with biotin-conjugated secondary antibodies (1:200, Jackson ImmunoResearch) and ABC VectaStain Elite (1:50, Vector Labs), followed by detection

with colorimetric metal-enhanced diamino-benzidine (1:10, ThermoScientific) or fluorescent TSA (1:50, Perkin-Elmer) (Lagace et al., 2007; Ables et al., 2010). Fluorescent sections were counterstained with nuclear 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI, 1:5000, Roche Applied Science). SM sections stained with DAB were counterstained with Nuclear Fast Red (Vector) and were dehydrated and coverslipped using DPX (Fisher Scientific). FF sections were mounted onto uncharged slides after IHC, and coverslipped using a glycerol-based anti-fade PVA-DABCO (reagents from Sigma).

Quantification of immunoreactive (+) cells

Quantification of BrdU+ and YFP+ cells in the SGZ of Nestin-Cre/YFP mice was performed with an Olympus BX-51 microscope (400x) with an area sampling fraction of 1, height sampling fraction of 1, and series sampling fraction of 9 (Lagace et al., 2007; Mandyam et al., 2007; Ables et al., 2010; Lagace et al., 2010). Quantification of GLAST::Cre/YFP+ GCL cells was performed with an Olympus BX-51 microscope (400x) via the optical fractionator method with a disector volume of $2.50 \times 10^{-5} \text{ mm}^3$ across a sampling grid of 0.0298 mm^2 and a series sampling fraction of 9, using StereoInvestigator. For Nestin-Cre/YFP mice, YFP+ RGCs were determined based on morphology (Ables et al., 2010), which included assessment of an irregular/triangular shaped cell body, thin wavering process headed through the GCL, and fine, filigree-like processes terminating in the boundary between the GCL and molecular layer.

Phenotypic analysis of BrdU+ cells and RGCs in NestinGFP mice

For assessment of dividing RGCs in NestinGFP mice, a 1:4 series of sections stained for BLBP/BrdU/GFP was analyzed from the anterior dentate gyrus until at least 500 BrdU+ cells were identified. BLBP+ RGCs were assessed for colocalization with NestinGFP and/or BrdU. RGCs were identified based on morphology.

Phenotypic analysis of YFP+ and NestinGFP+ cells

The neurogenic stages of YFP+ cells were assessed by phenotypic analysis of triple immunofluorescence labeling. At least 50 YFP+ or BrdU+ cells per mouse (n = 3 mice/group) were analyzed with a Zeiss LSM510 confocal microscope (emission wavelengths 488, 543, and 633, 630x in water) or Zeiss LSM510 confocal microscope (emission wavelengths 405, 488, 543, and 633, 400x in oil). Colocalization of signals was determined by scanning and optical sectioning in the Z plane. Neither the BrdU or YFP analyses were considered exhaustive, as BrdU and YFP cells in the same field were analyzed, but for BrdU-based analyses the analyses were "BrdU-biased," that is, YFP cells were analyzed only if they were identified in a field where BrdU+ cells were analyzed. Images of a single Z-plane were imported in Adobe Photoshop CS2 or CS5 and levels adjusted using the levels function for presentation purposes only. Three-dimensional reconstructions of cells from NestinGFP mice were prepared with LSM510 software from complete Z stacks collected at optimal pinhole diameters for each wavelength.

Statistical analyses

Data are reported as mean $\pm$ S.E.M. Statistical analyses were performed using unpaired *t*-tests or One- or Two-Way analysis of variance (ANOVA) followed by post-hoc analyses with the Bonferroni comparison as appropriate. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p < 0.05$.

Results

Hippocampal RGCs and progenitors provide distinct contributions to adult neurogenesis in nestin vs. GLAST Cre-driver lines

To explore the contribution of RGCs and progenitor cells to neurogenesis, we used two lines of TAM-inducible Cre-recombinase mice crossed to Rosa26R:YFP reporter mice (Fig. 1). There are no unique markers for hippocampal RGCs. Therefore we chose nestin and GLAST as markers because both are expressed in RGCs and RGCs are the only cell type where nestin and GLAST expression overlap (Fig. 1a)(Namba et al., 2005; Roybon et al., 2009). Nestin is also expressed in early neural precursor cells and GLAST is predominantly expressed in astrocytes. Following TAM administration, YFP⁺ cells are evident in the dentate gyrus SGZ of both transgenic lines, which allows us to characterize the progeny of stem-like cells by marker colocalization. For example, by staining for BLBP and Sox2, we could distinguish RGCs as BLBP+Sox2⁺ with radial morphology (Fig. 1b, arrow) and early progenitor cells as BLBP+Sox2⁺ with compact morphology (Fig. 1b, hatched arrows). We also identified neurons by colocalization with mature neuronal marker NeuN (Fig. 1c, arrowheads). In both Nestin-Cre/YFP and GLAST::Cre/YFP lines (Fig. 1d, g), we observed recombined YFP⁺ RGCs and early neuronal progenitor cells; we also observed immature and mature YFP⁺ dentate gyrus neurons, as shown previously (Lagace et al., 2007; Ninkovic et al., 2007; Ables et al., 2010). Thus, in keeping with prior work, administration of TAM to both lines of mice allowed us to label dentate gyrus progenitor cells (including RGCs) and track the number and phenotype of labeled progeny over time, enabling us to compare the addition of YFP⁺ cells between lines.

Our analysis revealed that an initial surge of YFP⁺ cells in Nestin-Cre/YFP mice 30d post-TAM was followed by a plateau in YFP⁺ cell number 60–180d post-TAM (Lagace et al., 2007)(Fig. 1e; $F_{(4,21)}=9.576$; $p<0.001$ with Bonferroni post-hoc showing significantly more YFP⁺ cells 30d vs. 12d, 60d, 100d, and 180d post-TAM). When we analyzed the phenotypes of YFP⁺ cells in Nestin-Cre/YFP mice (Fig. 1f) by colocalization with a panel of markers, most YFP⁺ cells 12–60d post-TAM were early progenitors (e.g. Sox2+/GFAP- or Sox2+/BLBP+ cells with non-radial morphology) (Lagace et al., 2007), while YFP⁺ cells 100–180d post-TAM were almost exclusively either neurons (YFP+NeuN+) or RGCs (YFP+BLBP+Sox2+ and YFP+GFAP+Sox2+ cells with radial morphology). We interpret these data to mean that in our Nestin-Cre^{ERT2} mouse, we label RGCs as well as a bolus of progenitor cells that expand, which accounts for the increase in YFP⁺ cell number through 30d. At later time points, this bolus of YFP⁺ progenitor cells differentiates into neurons, and ultimately some of these cells survive and integrate into hippocampal circuitry.

In contrast to Nestin-Cre/YFP mice, YFP⁺ cells continued to accumulate in GLAST::Cre/YFP mice, with significantly more YFP⁺ cells 180d than 12d post-TAM (Fig. 1h; $F_{(3,13)}=4.014$; $p<0.05$ with Bonferroni post-hoc showing significantly more YFP⁺ cells in GLAST::Cre/YFP mice 180d vs. 12d post-TAM). Phenotypic analysis of YFP⁺ cells in GLAST::Cre/YFP mice (Fig. 1i) revealed similarities in phenotypes compared to Nestin-Cre/YFP mice. Specifically, 12d and 30d post-TAM, most YFP⁺ cells were RGCs (YFP+BLBP+Sox2+ radial morphology) and early progenitor cells (YFP+BLBP+Sox2+ non-radial morphology), whereas at later times 100d and 180d post-TAM, many cells matured into neurons (YFP+NeuN+) with corresponding decreases in the proportions of RGCs (YFP+BLBP+Sox2+) and progenitors (Sox2+GFAP- or Sox2+BLBP+ non-radial cells). These data show that YFP⁺ RGCs and progenitor cells contribute to neurogenesis in both Nestin-Cre/YFP and GLAST::Cre/YFP mice, as YFP⁺ cells from both lines become neurons (Fig. 1f,i). Importantly, because GLAST::Cre/YFP also drives recombination in astrocytes, there were considerably more YFP⁺ cells labeled in GLAST::Cre/YFP than in Nestin-Cre/YFP mice. In addition, cells were quantified between lines using different techniques (*see*

Methods). Even so, YFP+ cells in GLAST::Cre/YFP mice continue to accumulate, while those in Nestin-Cre/YFP mice plateau (Fig. 1e,h).

To explore potential differences in the progenitor pools labeled in Nestin-Cre/YFP and GLAST::Cre/YFP mice, BrdU was administered 2h before sacrifice in mice that were 12d or 100d post-TAM (Fig. 1j). The proportion of BrdU+ cells that were YFP+ differed significantly between mouse lines ($F_{(1,10)}=7.324$; $p<0.05$), and there was a significant Strain x Time interaction ($F_{(1,10)}=11.55$; $p<0.01$). In Nestin-Cre/YFP mice – but not GLAST::Cre/YFP mice – a smaller proportion of BrdU+ cells were YFP+ between 12d and 100d post-TAM (Fig. 1k). Together, these results suggest differences in the dynamics of RGCs and progenitor cells labeled in Nestin-Cre/YFP versus GLAST::Cre/YFP mice.

YFP+ RGCs in GLAST::Cre/YFP – but not Nestin-Cre/YFP – mice contribute to neurogenic recovery after ablation

Our data (Fig. 1) suggested differences in RGC and/or progenitor cell contribution in Nestin-Cre/YFP versus GLAST::Cre/YFP mice in the basal state. Importantly, under baseline conditions, recombination in both transgenic lines labels RGCs as well as progenitors with YFP soon after TAM administration. This made it difficult to assess the specific contribution of YFP+ RGCs to ongoing neurogenesis. Therefore, to explore if YFP+ RGCs in both lines give rise to progenitors, we ablated proliferating progenitors using AraC, which stimulates RGCs to proliferate and repopulate the neurogenic niche (Seri et al., 2001). Specifically, Nestin-Cre/YFP and GLAST::Cre/YFP mice were given TAM to induce recombination, then received saline or AraC (i.c.v. over 6d) 25d post-TAM (Fig. 2a). Half of the animals in each group was examined 0d post-infusion to confirm that dividing cells were successfully ablated. The remaining half of animals was examined 7d post-infusion to assess recovery of the proliferative population. We chose to administer AraC at this time point because 30d post-TAM represents the peak in total YFP+ cells in Nestin-Cre/YFP mice (Fig. 1 and (Lagace et al., 2007)). Thus, AraC-mediated ablation 30d post-TAM would likely eliminate the greatest number of YFP+ cells. Further, when we performed surgery at an earlier time point (12d post-TAM), we encountered higher mortality, perhaps because of residual effects of TAM/EtOH/sunflower oil injection (data not shown).

AraC infusion significantly decreased the number of BrdU+ cells 0d post-infusion in both Nestin-Cre/YFP (Fig. 2b–c) and GLAST::Cre/YFP mice (Fig. 2g–h). Consistent with previous reports (Seri et al., 2001; Mak et al., 2007), the number of BrdU+ cells normalized within 7d post-infusion (Fig. 2c,h). This confirmed that AraC temporarily ablated proliferating cells in both lines of mice. To explore whether labeled RGCs contributed to the dividing cell pool in both Nestin-Cre/YFP and GLAST::Cre/YFP mice, we measured the total YFP+ cell number and the proportion of BrdU+ cells that were YFP+ RGCs (Fig. 2d–e,i–j). In Nestin-Cre/YFP mice, there were significantly fewer YFP+ cells post-AraC ($F_{(1,18)}=21.53$; $p<0.001$), with fewer YFP+ cells both 0d and 7d post-AraC vs. post-Sal (Fig. 2d). In Nestin-Cre/YFP mice, there was no change in the proportion of YFP+ RGCs that were dividing 7d post-infusion (Fig. 2e). One reason that there is no recovery of YFP+ progenitor cells in the Nestin-Cre/YFP mice could be that there are no more RGCs. However, when we quantified the number of YFP+ RGCs, we observed no change in the number of YFP+ RGCs as a result of infusion (Fig. 2f).

In contrast to Nestin-Cre/YFP mice, GLAST::Cre/YFP mice showed a significant effect of AraC infusion (Fig. 2i; $F_{(1,12)}=7.54$; $p<0.05$), with a trend towards fewer YFP+ cells 0d post-AraC vs. post-Sal ($p=0.05$). These results do not mean that AraC did not ablate proliferation in GLAST::Cre/YFP mice. Rather, these results suggest that at 30d post-TAM a much lower proportion (~15%) of YFP+ cells are early progenitors in GLAST::Cre/YFP vs. Nestin-Cre/YFP mice (Fig. 2d vs. 2i). However, 7d post-infusion the number of YFP+ cells was not

different between AraC- and saline-infused GLAST::Cre/YFP mice (Fig. 2i). Notably, there were significantly more dividing RGCs (BrdU+YFP+ with radial morphology) 7d post-infusion in GLAST::Cre/YFP mice infused with AraC vs. Sal controls (Fig. 2j). Importantly, because GLAST::Cre/YFP mice also label astrocytes, we needed to confirm that new YFP+ cells were indeed neurogenic. To this end, we found a significant increase in the proportion of YFP+ cells that were early neuronal precursor cells (Tbr2+Sox2+; Fig. 2k; $F_{(2,6)}=8.521$; $p<0.05$) (Steiner et al., 2006; Roybon et al., 2009). These results suggest YFP+ RGCs in GLAST::Cre/YFP mice contribute to neurogenic recovery 7d after AraC-induced ablation, whereas RGCs labeled in Nestin-Cre/YFP mice do not.

YFP+ RGCs in GLAST::Cre/YFP – but not Nestin-Cre/YFP – mice contribute to proliferation after stimulation

Thus far we identified differences in the Nestin-Cre/YFP and GLAST::Cre/YFP lines in the basal state and during proliferation recovery following chemically-induced ablation of dividing cells. To further test if YFP+ RGCs in both lines produce progenitors in a physiologically relevant model, we exposed mice to running wheels (Fig. 3a) to increase both neurogenesis and proliferation of RGCs (Ninkovic et al., 2007; Suh et al., 2007; Lugert et al., 2010). We reasoned that if labeled RGCs contribute to neurogenesis, then running should increase YFP+ cell number in both Nestin-Cre/YFP and GLAST::Cre/YFP mice. Importantly, running also increases the proliferation of early progenitor cells (Steiner et al., 2004; Lagace et al., 2007; Ables et al., 2010). Therefore, to specifically test whether YFP+ RGCs could give rise to YFP+ progeny, we started our running experiments 125d post-TAM, a time point when there were very few YFP+ early progenitor cells in either Nestin-Cre/YFP or GLAST::Cre/YFP lines (Fig. 1f,i). As expected, 14d of running stimulated proliferation, as the number of BrdU+ cells increased significantly in mice from both lines (Fig. 3b–d). Interestingly, in Nestin-Cre/YFP mice, 14d of running 125d post-TAM did not change the number of YFP+ cells (Fig. 3e). However, in GLAST::Cre/YFP mice, 14d of running 125d post-TAM significantly increased YFP+ cell number (Fig. 3e–g). These data suggest YFP+ cells – like RGCs – in GLAST::Cre/YFP mice can be stimulated to divide after 14d of running. In contrast, YFP+ cells in Nestin-Cre/YFP mice do not contribute to running-induced neurogenesis at a time (125d post-TAM) when the initial cohort of YFP+ progenitors is exhausted.

At least two subpopulations of hippocampal RGCs are neurogenic

Our data so far show differences in the dynamics of YFP+ cell pools labeled in inducible fate-tracking lines (Nestin-Cre/YFP and GLAST::Cre/YFP) under basal and stimulated conditions. While these data suggest differences in how YFP+ RGCs contribute to neurogenesis, it is also possible that YFP in these two lines labels different types of RGCs. Previous work indicated heterogeneity in RGCs, the proposed source of hippocampal neurogenesis (Kempermann et al., 2004; Steiner et al., 2006; Kim et al., 2007; Seki et al., 2007). We used transgenic reporter NestinGFP mice (Yamaguchi et al., 2000) to expand on these previous observations that SGZ RGCs are not a homogenous pool of cells, and to give more depth to our findings shown in Figs. 1–3. NestinGFP mice constitutively label cells expressing nestin with GFP, including RGCs and early progenitor cells (Fig. 1a). Because GFP fills the cells (process and cell body) whereas nestin protein is found largely within the radial process of RGCs (Fig. 4d), we used GFP expression as a reporter of nestin protein expression.

Within NestinGFP mice, we found RGCs could be categorized into two major subpopulations: RGCs that were NestinGFP+nestin+ versus NestinGFP+nestin- (Fig. 4a–d). These observations are based on immunostaining for a number of putative RGC markers, including GFAP, Sox2, BLBP, GLAST, and nestin (Fig. 1a) (Hartfuss et al., 2001; Seri et al.,

2001; Anthony et al., 2004; Bachoo et al., 2004; Kempermann et al., 2004; Seri et al., 2004; Namba et al., 2005; Encinas et al., 2006; Mori et al., 2006; Steiner et al., 2006). In general, we assessed two markers plus GFP (as the surrogate for nestin expression in NestinGFP mice). For example, we found radial GLAST+GFAP+ cells that were NestinGFP+ (Fig.4a, arrowheads) and some that were NestinGFP- (Fig.4a, arrows), suggesting that not all cells with radial morphology expressed NestinGFP. Using another pair of markers for stem-like cells, we also found BLBP+Sox2+ radial cells that were NestinGFP+ (Fig.4b, arrowheads) and NestinGFP- (Fig.4b, arrows). To test the possibility that RGCs lacking detectable nestin expression are mature astrocytes, we stained for S100 β . GFAP+NestinGFP- radial cells were negative for S100 β (Fig.4c, arrows), which is consistent with previous findings and suggests these RGCs are not mature astrocytes (Seri et al., 2004; Platel et al., 2009). To confirm the fidelity of the NestinGFP transgenic mice, we labeled endogenous nestin protein with NestinGFP and still observed GFAP+NestinGFP- nestin- RGCs (Fig.4d, arrows). Thus, using NestinGFP mice, we confirmed prior work that RGCs present radial morphology, co-expressed stem cell markers, and are antigenically heterogeneous (that is, some were nestin+; others were nestin-).

To further explore the extent to which nestin+ and nestin- subpopulations of RGCs are mitotically active and to quantify the mitotic potential of SGZ nestin+ and nestin- RGCs, 10-week-old NestinGFP mice were given multiple injections of BrdU (5x injections, 6h apart for 24h) to label cells in the S-phase of the cell cycle (Fig.4e). We stained for BrdU, NestinGFP, and BLBP, and assessed colocalization. When we considered all RGCs (BLBP+ cells with radial morphology, and NestinGFP+ cells with radial morphology), a very low proportion of RGCs was BrdU+ (Fig.4e, white bar). Conversely, when we considered all BrdU+ labeled cells, a very low proportion of BrdU+ cells was RGCs (BLBP+ with radial morphology, or NestinGFP+ with radial morphology; Fig.4e, black bar). This is consistent with previous reports (Steiner et al., 2006), and with similar observations we have seen with GFAP staining, where we very rarely observed BrdU+GFAP+NestinGFP+ RGCs cells (Fig. 4f). We then quantified the proportion of BLBP+ RGCs that were NestinGFP- (Fig.4e, hatched bar). Interestingly, we found a far greater proportion of BLBP+ RGCs that were NestinGFP-. However, we found no NestinGFP+ RGCs that were BLBP-. These observations of NestinGFP+ and NestinGFP- RGCs are consistent with our other observations (Fig.4a–d).

With two pools of RGCs (BLBP+NestinGFP+ or BLBP+NestinGFP-), we then used acute BrdU injection to ask: which RGCs are dividing? Almost all BrdU+ RGCs (>99%) were BLBP+NestinGFP+. In fact, analysis of >2000 total BrdU+ cells (500 cells from four mice) revealed only 3 BrdU+BLBP+NestinGFP- RGCs, constituting 0.14% of total BrdU+ cells and 0.13% of total RGCs. These data indicate that almost all dividing RGCs are BLBP+NestinGFP+, but also suggest that some BLBP+NestinGFP- RGCs may divide.

Discussion

We compared how YFP+ labeled cells in two widely-used inducible transgenic mouse lines – Nestin-CreER^{T2}/R26R:YFP and GLAST::CreER^{T2}/R26R:YFP – contribute to adult hippocampal neurogenesis. While these two lines have been well-described in a number of publications, methodological differences between reports prevented generalized conclusions about the labeled pools of RGCs and progenitor cells, and encouraged us to perform the direct comparisons reported here. After exploring the basal contribution of labeled RGCs to constitutive neurogenesis, we manipulated neurogenesis (chemically ablating progenitor cells with AraC, and increasing proliferation via running) to assess the neurogenic contribution of labeled RGCs. While we find similarities between lines basally, we also find some key differences that support RGC heterogeneity. Importantly, after running or

ablation, we find that labeled RGCs in GLAST::CreER^{T2} mice contribute to neurogenesis, whereas labeled RGCs in Nestin-CreER^{T2} mice do not. To support our conclusions from the two inducible fate-tracking transgenic mouse lines, we also used the NestinGFP mouse line to highlight the heterogeneity of RGCs *in vivo*.

As discussed below, these data highlight the challenges in comparing transgenic lines where RGC and progenitor cell behavior and contribution to neurogenesis may differ depending on the driver employed. These data also raise questions about RGC and progenitor cell heterogeneity, and the potential differential contribution of nestin+ and GLAST+ cells to neurogenesis. Therefore, these data emphasize that careful assessment of the accepted “milestones of neurogenesis” is warranted, and encourage the field to reevaluate the assumption each RGC is a putative source of adult-generated hippocampal neurons *in vivo*.

Comparison of inducible transgenic mouse lines basally

Our data with inducible fate mapping highlight similarities and differences between Nestin-CreER^{T2}/R26R:YFP and GLAST::CreER^{T2}/R26R:YFP mice. For example, both lines support neurogenesis: there are no YFP+ neurons at early times, but a gradual accumulation of YFP+ neurons with time. On the other hand, there are striking differences between the pools of cells labeled in these Cre-driver lines. For example, in the Nestin-Cre/YFP mouse, we label a bolus of YFP+ cells that peaks around 30d post-TAM, after which the number of YFP+ cells stabilizes at time points (60–180d). In the GLAST::Cre/YFP line, on the other hand, we similarly observe YFP+ RGCs and progenitor cells which mature into neurons, but interestingly, the total number of YFP+ cells continues to increase over time, even 180d post-TAM. This correlates with an increase in the proportion of cells that present a neuronal phenotype. We interpret these data to mean we have labeled stem and progenitor cells that continuously contribute to neurogenesis over time. An alternative explanation of our results is that the two lines either label different cell pools or have different efficacy in labeling cells; these possibilities are discussed in detail below and warrant further investigation.

We have previously reported that 97% of GFAP+Sox2+ RGCs were labeled in Nestin-Cre/YFP mice at 12d post-TAM (Lagace et al., 2007). It is possible that GFAP+ RGCs which lack nestin expression might not express Sox2. Alternatively, it is possible that our staining techniques and analysis methods did not allow us to clearly appreciate nestin+ and nestin-subsets of RGCs. For example, GFP staining in the NestinGFP mouse is much brighter than the YFP detection in Nestin-CreER^{T2}/R26R:YFP mice. Further work is ongoing to explore these possibilities.

Comparison of inducible transgenic mouse lines: perturbation of neurogenesis

To further explore differences and similarities in how cells labeled in Nestin-CreER^{T2}/YFP and GLAST::CreER^{T2}/YFP mice contribute to neurogenesis, we used AraC and running to ablate or stimulate proliferation, respectively. We hypothesized factors that modulate neurogenesis would coincidentally change the pools of YFP+ cells. Indeed, as expected the YFP+ cells in the GLAST::Cre/YFP mice were able to respond to two distinct neurogenic stimuli: running and AraC-mediated ablation of progenitors. More specifically, we found increased cell division of YFP+ RGCs in GLAST::Cre/YFP mice after AraC, demonstrating a role for RGCs in neurogenic recovery. In contrast, it was surprising that YFP+ RGCs in Nestin-Cre/YFP mice were unable to contribute to either running- or ablation-stimulated proliferation, since in YFP+ RGCs were present and unchanged in number. This suggests that the commonly used phrase “nestin+ stem cell” may be an oversimplification of the *in vivo* contribution of nestin+ cells to ongoing neurogenesis.

Evidence for functional heterogeneity of RGCs, or evidence for different *in vivo* progenitor cell behavior in nestin vs. GLAST lines?

What might explain our data showing differences between basal and perturbed neurogenesis between inducible transgenic lines? These data might highlight a functional heterogeneity between stem cell populations. In fact there are several lines of evidence that support the interpretation that nestin and GLAST lineage RGCs differ in their contribution to adult hippocampal neurogenesis, and the data presented here offer new interpretations of data already present in the literature using other nestin-transgenic mouse lines. For example, there are differing reports on recovery following inducible ablation of neurogenesis using ganciclovir in thymidine kinase mouse lines. There was no recovery of neurogenesis in GFAP-tk mice (Garcia et al., 2004), whereas there was at least partial recovery in nestin-tk mice despite the persistence of RGCs (Singer et al., 2009). This may reflect differences in neurogenic potential between GFAP+ and nestin+ populations. In another Nestin-CreER^{T2} mouse line, repeated rounds of TAM administration were required for maximal recombination, despite very high recombination efficiencies after a single round (Imayoshi et al., 2008). This may be due to the possibility that nestin-expressing cells are not the constitutive source of neurogenesis. In light of our current data, these previous reports could be reinterpreted to support functional heterogeneity between nestin+ and GFAP+GLAST+ cells within the RGC population in the adult (Steiner et al., 2006; Kim et al., 2007; Seki et al., 2007; Liu et al., 2010). Further, our evidence that RGCs divide a limited number of times before becoming quiescent fits with other recent publications (Bonaguidi et al., 2011; Encinas et al., 2011).

While our data support functional heterogeneity of RGCs, alternative explanations are important to consider. Our current data may be the result of different efficiencies in labeling the stem cell source of hippocampal neurogenesis. On a related point, these data may also be the result of different progenitor cell “behaviors” in nestin and GLAST lineage cells; for example, perhaps labeled progenitors in the GLAST line divide faster and thus repopulate the SGZ more rapidly than labeled progenitors in the nestin line. These and other alternative explanations have been challenging to evaluate. Concurrent assessment of the contributions of GLAST and nestin lineage cells to neurogenesis *in vivo* would require new technologies, such as advances in intersectional fate tracking using inducible split-Cre approaches (Beckervordersandforth et al., 2010) or combination of Nestin-CreER^{T2} and inducible GLAST- or GFAP-FlipaseER^{T2}. When these approaches are widely available, they will be critical in further dissection of radial glia heterogeneity, direct assessment of the lineage of GLAST and nestin subpopulations, and thus permit evaluation of these alternative explanations. However, we feel that our current data minimally encourage broad reconsideration that there are functionally distinct stem cell populations in the adult hippocampus.

Implications for the field of adult neurogenesis

While additional study is needed to assess to what extent nestin and GLAST lineage RGCs contribute to adult hippocampal neurogenesis *in vivo*, the present data and other recent publications (Bonaguidi et al., 2011; Dranovsky et al., 2011; Encinas et al., 2011; Bonaguidi et al., 2012) present conflicting data regarding the role of RGCs in continuous neurogenesis. For example, the results presented here with the Nestin-CreER^{T2}/R26R:YFP mouse suggest that labeled RGCs likely divide a limited number of times, producing a pool of progenitor cells but then exhaust. This is largely consistent with results from Encinas and colleagues, who show that RGCs divide up to three times in rapid succession, then ultimately exhaust (Encinas et al., 2011); among multiple inducible fate-tracking lines used, the authors also used a different Nestin-CreER^{T2} mouse. Our observed results are also consistent with other results with a different Nestin-CreER^{T2} mouse in which clonal analysis was used to show

that most RGCs produce only a few cells (either astrocytes or neurons) but do not continuously produce progeny (Bonaguidi et al., 2011). Interestingly, the results we observe and the results observed by both groups fit with a limited role of nestin-expressing RGCs in hippocampal neurogenesis (Bonaguidi et al., 2012). On the other hand, our data with GLAST::CreER^{T2}/R26R:YFP mice fit with some observations made by Rene Hen's group with a different Nestin-CreER^{T2} line (Dranovsky et al., 2011). Specifically, the continued expansion of the labeled YFP+ cell pool over time in their mouse suggests some nestin-labeled cells may be a continuous source for constitutive adult hippocampal neurogenesis.

Taken together, our results and these other seminal papers urge the field to re-think the current model of hippocampal neurogenesis. For example, one consideration is that there may be endogenous differences between nestin+ and GLAST+ RGCs, where nestin-expressing RGCs represent more “short-term” stem cells, whereas GLAST+/nestin- RGCs may be “long-term” stem cells. More specifically, when our results comparing GLAST::CreER^{T2}/Rosa26:YFP and Nestin-CreER^{T2}/Rosa26:YFP lines are considered with our data that nestin+ RGCs comprise over 99% of dividing RGCs, a tempting hypothesis emerges: GLAST+/nestin- RGCs are largely dormant, but when activated (potentially through cell intrinsic factors like Notch1 (Ables et al., 2010)), GLAST+/nestin- RGCs become nestin+ and then produce progeny. Nestin+ RGCs may then only divide a limited number of times prior to losing proliferative potential. This hypothesis that RGCs divide then senesce could help explain the age-related decrease in neurogenesis (Kuhn et al., 1996; Seki et al., 2007; Bonaguidi et al., 2011; Encinas et al., 2011), and suggests the gradual depletion of RGCs that are capable of dividing underlies reduced neurogenesis with age. It also explains why we observe a persistence of labeled RGCs after AraC, but no recovery of neurogenesis in the Nestin-Cre/YFP line. While recent reports have suggested that post-mitotic RGCs differentiate into astrocytes (Encinas et al., 2011), we find almost no YFP+ astrocytes in the SGZ (Lagace et al., 2007) and the number of YFP+ RGCs remains constant over time. It is possible, however, that newly generated astrocytes migrate into adjacent regions, such as the hilus, or remain as quiescent radial astrocytes in the dentate gyrus.

Our data also suggest differences between nestin+ and GLAST+ RGCs in their ability to contribute to neurogenesis over long time, and urge us to revisit stem cell function in the adult hippocampus. Our data fit well with other recent manuscripts suggesting a limited contribution of RGCs to long-term neurogenesis (Encinas et al., 2011), and emphasize a need to re-evaluate fundamental assumptions regarding adult neurogenesis before we can harness neural stem cells for regenerative medicine and other clinical applications.

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Figure 1. Dynamic contribution to hippocampal neurogenesis in inducible Nestin-Cre/YFP and GLAST::Cre/YFP mice

(a) Schematic representation of hippocampal neurogenesis, including cell types (top). The transgenic mice lines used in current studies have partially overlapping cell targets, specifically within the RGC pool. Colored bars represent approximate cellular expression of antigenic markers used in current studies to phenotype cells. **(b–c)** Phenotypic analysis of RGCs and progenitor cells. **(b)** Representative section from Nestin-CreER^{T2}/R26R:YFP mouse dentate gyrus (DG) 30d post-tamoxifen (TAM). Arrow: YFP+/BLBP+/Sox2+ RGC with characteristic irregularly-shaped soma and central process extending into GCL. Hatched arrows: YFP+/BLBP+/Sox2+ early progenitor cells with compact somal

morphology. Arrowhead: YFP+/BLBP-/Sox2- cell. Scale bar in **(b)**=20 μ m. **(c)** Phenotypic analysis of mature neurons in GLAST::CreER^{T2}/R262R:YFP mouse DG 30d post-TAM. Arrow: YFP+/NeuN-/GFAP+ RGC with characteristic irregularly-shaped soma and radial process. Arrowheads: YFP+/NeuN+/GFAP- mature neurons. Scale bar in **(c)**=15 μ m. **(d, g)** Schematic representation of **(d)** Nestin-CreER^{T2}/R26R:YFP (“Nestin-Cre/YFP”) and **(g)** GLAST::CreER^{T2}/R26R:YFP (“GLAST::Cre/YFP”) mice. **(e, h)** Total number of YFP+ cells in **(e)** Nestin-Cre/YFP or **(h)** GLAST::Cre/YFP mice at various times post-TAM. The data in **(e–f)** include previously published observations by Lagace et al., 2007 (indicated on graphs). Mean $\pm$ SEM, $n=3–6$ mice/group, * $p<0.05$ vs. 12d post-TAM, One-Way ANOVA with Bonferroni post-test. **(f, i)** Phenotypic analysis of YFP+ cells in **(f)** Nestin-Cre/YFP or **(i)** GLAST::Cre/YFP mice over time. Radial glia (blue square): YFP+Sox2+GFAP+ or YFP+Sox2+BLBP+ cells with radial morphology. Early progenitor (red circle): YFP+Sox2+GFAP- or YFP+Sox2+BLBP+ cells with compact morphology. Mature neuron (black triangle): YFP+NeuN+. Mean $\pm$ SEM, 51–178 YFP+ cells/mouse, $n=3$ mice/group. **(j–k)** *In vivo* YFP+ progenitor pool dynamics in Nestin-Cre/YFP and GLAST::Cre/YFP mouse lines. **(j)** Schematic representation of experimental design. BrdU was administered 2h before sacrifice 12d or 100d post-TAM. **(k)** The proportion of BrdU+ cells that are YFP+ in Nestin-Cre/YFP vs. GLAST::Cre/YFP mice 12d (white bars) or 100d (black bars) post-TAM. * $p<0.05$ by Two-Way ANOVA with Bonferroni post-test. Mean $\pm$ SEM, 30–109 BrdU+ cells/mouse, $n=3–4$ mice).

Figure 2. AraC-mediated ablation of proliferation and subsequent recovery in inducible mice (a) Schematic of experimental design. (b, g) Representative BrdU and YFP immunostaining with DAPI at 7d recovery post-infusion in (b) Nestin-Cre/YFP and (g) GLAST::Cre/YFP mice. Inset (g) highlights a BrdU+YFP+ RGC. Scale in (b)=100μm, and applies to (b, g). (c, h) BrdU+ cells in (c) Nestin-Cre/YFP and (h) GLAST:Cre/YFP mice after saline (white bars) or AraC (black bars). Data presented as percent of saline, 0d recovery, *** $p < 0.001$ vs. Saline control, Two-WAY ANOVA with Bonferroni post-test, Mean±SEM, $n = 3-8$ mice/group. (d, i) YFP+ cells in (d) Nestin-Cre/YFP and (i) GLAST::Cre/YFP mice after saline (white bars) or AraC infusion (black bars). Data presented as percent of saline, 0d recovery, Mean±SEM, $n = 3-8$ mice/group, # $p = 0.05$; * $p < 0.05$; ** $p < 0.01$ vs. Saline control, One-Way ANOVA with Bonferroni post-test. (e, j) Proportion of BrdU+ cells that are YFP+ with RGC morphology at 7d recovery post-infusion in (e) Nestin-Cre/YFP and (j) GLAST::Cre/YFP mice after saline (white bars) or AraC (black bars). Data presented as percent of BrdU cells, 7d recovery, Mean±SEM, $n = 50-71$ cells/mice, $n = 3-7$ mice/group, ** $p < 0.01$ vs. Saline control, t -test. (f) Quantification of YFP+ RGCs in Nestin-Cre/YFP after saline (white bars) or AraC infusion (black bars). (k) Proportion of YFP+ cells that are neuronal precursors (Tbr2+Sox2+) in GLAST::Cre/YFP mice after infusion with saline (7d recovery, white bars); AraC (0d recovery, black bars); or AraC (7d recovery, hatched bars). Data presented as percent of YFP+ cells, Mean±SEM, $n = 3$ mice/group, * $p < 0.05$; ** $p < 0.01$ vs. AraC, 0d, One-Way ANOVA with Bonferroni post-test. Arrowheads indicate representative Tbr2+Sox2+YFP+ cells. Scale in (k)=10μm.

Figure 3. Running increases YFP+ cell number in inducible GLAST::Cre/YFP but not Nestin-Cre/YFP mice
(a) Schematic of experimental design. **(b–d)** BrdU+ cells and **(e–g)** YFP+ cells after 14d access to locked (control, white bars) or open (running, black bars) wheels in Nestin-Cre/YFP and GLAST::Cre/YFP mice. **(b, e)** Quantification of **(b)** BrdU+ cells and **(e)** YFP+ cells. Data presented as Mean±S.E.M. of total cells **(b)** or percent of Locked Wheel control **(e)** ($n=3-8$ mice/group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, t -test). **(c–d, f–g)** Representative images of dentate gyri from GLAST::Cre/YFP mice with access to locked or open running wheels showing **(c–d)** BrdU+ cells and **(f–g)** YFP+ cells. Inset **(d)** highlights several clusters

of BrdU+ cells. **(c)** Scale bar in **(c)**=100μm, applies **(c–d)**; Inset in **(d)**= 20μm. Scale in **(f)**=100μm, applies **(f–g)**.

Figure 4. Antigenic and mitotic heterogeneity of RGCs in NestinGFP reporter mice
(a–d) Three dimensional reconstructions of confocal Z-stack images depicting marker expression in SGZ RGCs of adult NestinGFP mice. **(a)** Arrowheads: GLAST+GFAP+NestinGFP+ RGCs. Arrows: GLAST+GFAP+NestinGFP- RGCs. **(b)** Arrowheads: BLBP+Sox2+NestinGFP+ RGCs. Arrows: BLBP+Sox2+NestinGFP- RGCs. **(c)** Arrowheads: GFAP+NestinGFP+ RGCs. Arrows: S100 β -GFAP+NestinGFP- RGCs. Asterisks(*): S100 β +GFAP+ astrocytes found in DG granule cells layer/SGZ. **(d)** Arrowheads: GFAP+Nestin+NestinGFP+ RGCs. Arrows: GFAP+Nestin-NestinGFP- radial processes. Scale in **(a–d)**=20 μ m. **(e)** Experimental design and results for quantification of the mitotic potential of NestinGFP+ and NestinGFP- RGCs. Proportion of RGCs that were BrdU+ (white bar); proportion of BrdU+ cells that were RGCs (black bars); proportion of RGCs that were NestinGFP- (hatched bars). Data presented as percent, Mean $\pm$ S.E.M., n =500+ BrdU+ cells/mouse, n =4 mice/group. **(f)** Representative photomicrograph of a BrdU+GFAP+NestinGFP+ RGC. Scale in **(f)**=10 μ m.