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Characterization of neural stem cells and their progeny in the adult zebrafish optic tectum

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ABSTRACT

In the adult teleost brain, proliferating cells are observed in a broad area, while these cells have a restricted distribution in adult mammalian brains. In the adult teleost optic tectum, most of the proliferating cells are distributed in the caudal margin of the periventricular gray zone (PGZ). We found that the PGZ is largely divided into 3 regions: 1 mitotic region and 2 post-mitotic regions—the superficial and deep layers. These regions are distinguished by the differential expression of several marker genes: *pcna*, *sox2*, *msi1*, *elavl3*, *gfap*, *fabp7a*, and *s100β*. Using transgenic zebrafish Tg (*gfap*:GFP), we found that the deep layer cells specifically express *gfap*:GFP and have a radial glial morphology. We noted that bromodeoxyuridine (BrdU)-positive cells in the mitotic region did not exhibit glial properties, but maintained neuroepithelial characteristics. Pulse chase experiments with BrdU-positive cells revealed the presence of self-renewing stem cells within the mitotic region. BrdU-positive cells differentiate into glutamatergic or GABAergic neurons and oligodendrocytes in the superficial layer and into radial glial cells in the deep layer. These results demonstrate that the proliferating cells in the PGZ contribute to neuronal and glial lineages to maintain the structure of the optic tectum in adult zebrafish.

Introduction

Adult neurogenesis in the mammalian brain occurs in a restricted region of the telencephalon (Kempermann, 2006; Ming and Song, 2005). Previous studies have demonstrated that the ability to produce new neurons in the adult brain plays an important role in the maintenance of brain functions such as learning and memory (Clelland et al., 2009; Drapeau et al., 2003; Garthe et al., 2009; Kee et al., 2007). However, the underlying molecular mechanisms that regulate this phenomenon are largely unknown (Alvarez-Buylla and Lim, 2004). Non-mammalian vertebrates such as reptiles, amphibians, and teleosts retain proliferating cells outside of the telencephalon in the adult brain (Chapouton et al., 2007; Kaslin et al., 2008). In teleosts, including zebrafish (*Danio rerio*), the entire brain continues to grow in adulthood, and proliferating cells are still observed in a broad area of the rostrocaudal axis (Adolf et al., 2006; Bernardos et al., 2007; Chapouton et al., 2006; Grandel et al., 2006; Hinsch and Zupanc, 2007; Kaslin et al., 2009; Marcus et al., 1999; Raymond et al., 2006). Müller glia-derived progenitor cells generate rod photoreceptor lineage in the adult retina (Bernardos et al., 2007). Progenitors derived from the ventral subpallium in the adult telencephalon migrate into the olfactory bulb through the rostral migratory stream, and then differentiate into GABAergic or tyrosine hydroxylase (TH)-positive neurons (Adolf et al., 2006). The hairy-related 5 (*her5*)-positive cell population in the adult midbrain–hindbrain boundary differentiates into neurons and glia (Chapouton et al., 2006). In the adult cerebellum, neural stem cells possess neuroepithelial characteristics and produce granule cell precursors depending on fibroblast growth factor (Fgf) signaling (Kaslin et al., 2009). These studies demonstrated that progenitor cells in the adult zebrafish brain retain neural stem cell properties similar to those in the mammalian central nervous system (CNS). Therefore, the adult teleost brain is considered to be an excellent comparative model for adult neurogenesis in vertebrates (Chapouton et al., 2007; Kaslin et al., 2008).

The optic tectum is a visual center of the teleost brain and dominates the dorsal part of the mesencephalon, which corresponds to the superior colliculus of the mammalian midbrain. The optic tectum has a multilayered structure (from the superficial to the deep layer) consisting of the stratum marginale (SM), stratum opticum (SO), stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (SGC), stratum album centrale (SAC), and stratum periventriculare (SPV) (Meek, 1983; Meek and Nieuwenhuys, 1998). Most of the neurons of the optic tectum exist in the SPV layer, also called the periventricular gray zone (PGZ). Neurons in the PGZ extend their apical dendrites to the SO and SFGS layers and make glutamatergic synapses with retinal axons thereby receiving visual

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information (Kinoshita et al., 2005; Kinoshita and Ito, 2006). Proliferating cells have been identified in the PGZ of the optic tectum in adult teleosts such as the brown ghost knifefish (*Apteronotus leptorhynchus*), brown trout (*Salmo trutta fario*), three-spined stickleback (*Gasterosteus aculeatus L.*), goldfish (*Carassius auratus*), medaka (*Oryzias latipes*), and zebrafish (Candal et al., 2005; Ekström et al., 2001; Grandel et al., 2006; Hinsch and Zupanc, 2007; Marcus et al., 1999; Nguyen et al., 1999; Raymond and Easter, 1983; Zupanc and Horschke, 1995; Zupanc et al., 2005), suggesting continuous neurogenesis in the adult teleost optic tectum. In the optic tectum of adult zebrafish, proliferating cells exist in the medial, lateral, and caudal margins of the PGZ (Grandel et al., 2006; Marcus et al., 1999; Zupanc et al., 2005). However, there is no evidence whether these proliferating cells possess the molecular properties of neural stem cells.

In this study, we demonstrated that the proliferating cells in the adult zebrafish optic tectum function as neural stem/progenitor cells *in vivo*. We found that bromodeoxyuridine (BrdU)-labeled proliferating cells in the mitotic region of the PGZ expressed neural stem/progenitor cell markers such as *proliferating cell nuclear antigen* (*pcna*), *SRY-box containing gene 2* (*sox2*), *musashi homolog 1 (Drosophila)* (*msi1*) (Bravo and Macdonald-Bravo, 1987; Ferri et al., 2004; Kaneko et al., 2000). BrdU-negative cells located in the ventral edge of the PGZ, which we designated as deep layer cells, still expressed several neural stem/progenitor cell markers and some glial cell markers such as *glial fibrillary acidic protein* (*gfap*), *fatty acid binding protein 7, brain, a* (*fabp7a*, also called *brain lipid binding protein*, *blbp*), and *S100 calcium binding protein, beta* (*neural*) (*s100β*) (Götz and Barde, 2005; Hartfuss et al., 2001; Liu et al., 2003; Wainwright et al., 2004). Using a transgenic Tg (*gfap*:GFP) zebrafish strain (Bernardos and Raymond, 2006), we showed that these *gfap*–GFP-positive deep layer cells extended radial fibers, indicating that these cells are radial glia. We were intrigued to note that BrdU-positive proliferating cells did not exhibit glial properties, which are a common feature of neural stem cells in the adult mammalian brain. The proliferating cells that face the ventricle show a polarized distribution of apical markers, including zona occludens protein 1 (ZO-1), γ-tubulin, and aPKC (Del Bene et al., 2008; Oteiza et al., 2008), suggesting that these cells maintain neuroepithelial characteristics. Cell lineage tracing, with BrdU pulse labeling, revealed that these proliferating cells differentiated into the *ELAV* (*embryonic lethal, abnormal vision, Drosophila*)*-like3* (*Hu antigen C*) (*elavl3*, also called *Hu antigen C*, *huC*)-positive neuronal cells, which finally differentiated into glutamatergic or GABAergic neurons in the superficial layer of the PGZ (Higashijima et al., 2004; Martin et al., 1998; Marusich et al., 1994; Mueller and Wullimann, 2002), oligodendrocytes, and radial glial cells in the deep layer of the PGZ. Each cell type differentiated at least 2 weeks after the final division of their progenitors.

Materials and methods

Animals

Zebrafish (*D. rerio*) were bred and maintained according to standard procedures (Westerfield, 2007). RIKEN Wako (RW) wild-type strain was obtained from the Zebrafish National BioResource Center of Japan (http://www.shigen.nig.ac.jp/zebra/). The Tg (*gfap*:GFP)$^{mi200\ 1}$ (Bernardos and Raymond, 2006) strain was obtained from the Zebrafish International Resource Center (ZIRC). The Tg (*elavl3* (*huC*):GFP) (Park et al., 2000) strain was provided from the Lab. for Developmental Gene Regulation, BSI, RIKEN.

Bromodeoxyuridine labeling

Adult fish (age, 6–10 months; weight, 0.16–0.57 g; length, 28–40 mm) were anesthetized in fish water containing 0.017% tricaine (pH 7.0; Nacalai Tesque). They were then intraperitoneally injected with 16 mM bromodeoxyuridine (BrdU; Sigma) solution diluted in E3 medium with 50 μl/g body weight and kept in fish water containing 10 mM BrdU for 72 h. For the BrdU pulse chase in Figs. 6A–H, 24-hour-BrdU-labeled fish were incubated in fresh fish water for 2 weeks, 1 month, and 2 months. For the BrdU pulse chase in Figs. 6I–T and 7, 72-hour-BrdU-labeled fish were incubated in fresh fish water for 2 weeks (Figs. 6I–P) or 1 month (Figs. 6Q–T and 7). After incubation, the fish were placed on ice and decapitated. The brains were dissected and fixed in 4% paraformaldehyde (PFA; Wako) solution dissolved in phosphate-buffered saline (PBS, pH 7.4) at 4 °C for 24 h and then dehydrated gradually in ethanol and stored in 100% ethanol at −20 °C.

Bromodeoxyuridine and iododeoxyuridine double-labeling

BrdU and iododeoxyuridine (IdU; Sigma) double-labeling was performed according to Burns and Kuan (2005) with some modification. Intraperitoneal injection of 10 mM IdU at 80 μl/g body weight was administered to anesthetized adult fish, which were then maintained in fish water containing 10 mM IdU for 48 or 66 h. Intraperitoneal injection of 16 mM BrdU at 50 μl/g body weight was then administered, and the animals were maintained in fish water containing 10 mM BrdU for 24 or 6 h, for a total labeling time of 72 h for both samples.

Histology

For fluorescence *in situ* hybridization and immunohistochemistry, fish were anesthetized in 0.017% tricaine and perfused intracardially with Ringer's solution followed by 4% PFA solution. The brains were dissected from the skulls and postfixed in 4% PFA solution overnight at 4 °C. To prepare frozen sections, whole brains were soaked in 20% sucrose at 4 °C overnight and embedded in an embedding solution [O.C.T. compound (Tissue-Tek): 20% sucrose = 2:1]; 14-μm-thick sections were cut using a cryostat (Cryocut1800; Leica). For vibratome sections, whole brains were embedded in 2% agarose and 60-μm-thick sections were prepared using a micro slicer (DTK-1000, Zero1, Dosaka EM). Plastic sections were prepared for counting cell numbers; a whole brain, which was already stained with anti-BrdU and detected by Histofine simple stain MAX-PO (M) (Nichirei) (see below), was dehydrated gradually in ethanol and embedded using the JB-4 embedding kit (Polysciences). The brains were then cut into 10-μm-thick serial sections using a rotary microtome (HM330; Microm). The serial sections were mounted using Entellan (Merck).

Cell quantifications/cell counting

To quantify BrdU-positive cells, 10-μm-thick serial coronal plastic sections through the whole tectal region were prepared as described above ($n = 3$). BrdU-positive cells were counted on a BX50 microscope (Olympus) with UPlanFLN 60× (NA0.90) objectives. Since the size of the teleost brain is slightly different between samples of the same age, we divided all sections into 10 groups along the rostrocaudal axis and calculated the mean cell number of each group. This mean cell number was compared with the corresponding group of samples. Means were expressed ± SEM.

Immunohistochemistry

Immunohistochemistry was performed on 14-μm-thick cryosections and 60-μm-thick vibratome sections. Briefly, each sample were washed several times in 0.1% PBST (PBS containing 0.1% Triton X-100) and then blocked in 0.1% PBST with a 2% blocking regent (Roche) for 1 h at room temperature before application of the primary antibody. For primary antibodies, we used mouse anti-BrdU (1:100; Roche), rat anti-BrdU (1:500; Abcam), mouse anti-BrdU (1:500; Becton

Dickindon) (this antibody can detect both BrdU and IdU), mouse anti-PCNA (1:1000; Sigma), rabbit anti-PCNA (1:50; Santa Cruz), rabbit anti-phospho-histone H3 (pH3) (1:500; Upstate biotech), rabbit anti-Sox2 (1:200; Millipore), rabbit anti-Musashi 1–2 (1:1000; a gift from Dr. Michael Brand and Dr. Jan Kaslin, Dresden University of Technology, Germany) (Kaslin et al., 2009), mouse anti-HuC/D (1:40; Molecular Probes), mouse anti-GFAP (1:500; zrf-1, ZIRC), rabbit anti-S100 (1:500; Dako), rabbit anti-BLBP (1:1000; Abcam), mouse anti-ZO-1 (1:1000; Invitrogen), mouse anti-γ-tubulin (1:500; Sigma) and rabbit anti-aPKC (1:250; Santa Cruz), mouse anti-GFP (1:200; Roche), and rabbit anti-GFP (1:100; Santa Cruz). For secondary antibodies, we used Histofine simple stain MAX-PO (M) (Nichirei) and Alexa Fluor 350-, 488-, 546-, 594-, and 647-conjugated subclass-specific antibodies (1:500; Invitrogen). Sections were embedded in PermaFluor (Thermo) or 70% glycerol. For immunodetection of BrdU, the samples were incubated in 2 M HCl for 30 min at 37 °C before blocking. For immunodetection of PCNA, antigen retrieval was performed by incubating slides in 10 mM sodium citrate for 30 min at 90 °C before treatment with the primary antibody. For immunodetection of HuC/D, the slides were incubated in methanol prior to treatment with the primary antibody. For nuclear staining, the samples were incubated in SYTOX orange (1:3000; Invitrogen) for several minutes after immunohistochemistry was performed.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization was performed according to a previously described method with some modifications (Takahata et al., 2006). Briefly, 14-μm-thick frozen sections were washed in 0.3% H_2O_2 in methanol for 30 min at room temperature, followed by a brief wash in sterile water. After washing in PBST, the sections were treated with 1 μg/ml proteinase K in PBST for 7 min at 37 °C and postfixed in 4% PFA in PBS. After washing in PBST, the sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 15 min at room temperature and washed in standard saline citrate (SSC). For prehybridization, the sections were incubated in a hybridization buffer (5× SSC, 2% blocking reagent, 50% formamide, 0.1% N-lauroylsarcosine (NLS), and 0.1% SDS; pH 7.0) for 1 h at 65 °C. This was replaced with the hybridization buffer containing 1.0 μg/ml DIG-labeled RNA probes and incubated at 65 °C overnight. After hybridization, sections were washed twice in 2× SSC containing 50% formamide and 0.1% NLS for 20 min at 65 °C, and excess RNA probes were digested in an RNase A buffer (10 mM Tris–HCl, 10 mM EDTA, and 0.5 mM NaCl containing 20 μg/ml RNase A) for 15 min at 37 °C. The slides were washed twice in 2× SSC/0.1% NLS for 20 min at 37 °C and in 0.2× SSC/0.1% NLS for 15 min at 37 °C. After blocking in 2% blocking solution in PBST for 1 h at room temperature, the slides were incubated overnight in anti-digoxigenin-horse radish peroxidase (DIG-POD) Fab fragments (1:100; Roche) at 4 °C. To detect the POD-labeled antibody, the signal was enhanced by the tyramide signal amplification (TSA) plus dinitrophenyl (DNP) system (PerkinElmer) and visualized by an Alexa Fluor 488-conjugated anti-DNP-KLH antibody (1:500, Molecular Probes). After visualization, immunohistochemistry for BrdU detection was performed as described above. In addition to the cDNA clones that we independently isolated (as described below), we also used the following plasmids as templates to synthesize digoxigenin-labeled RNA probes: *solute carrier family 17* (*sodium-dependent inorganic phosphate cotransporter*) *member 6a* (*slc17a6a*, also called *vglut2.2*) (Higashijima et al., 2004), *slc17a6b* (*slc17a6b*, also called *vglut2.1*) (Higashijima et al., 2004), *glutamate decarboxylase (gad) 1* (Martin et al., 1998), and *gad2* (Martin et al., 1998).

cDNA cloning

Total RNA was extracted from the whole brain of adult zebrafish using a total RNA extraction kit (RNAiso Plus, TaKaRa), and cDNA was synthesized using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). The RT-PCR-amplified fragments were cloned into pCRII-TOPO plasmids (Invitrogen). The following primer sets were used to amplify coding sequence from cDNA: *fabp7a* sense: 5′-TGTTTCATCATCTCTCAACATGGTC-3′ and antisense: 5′-GGTAATACT-GAAACGTCCTGCGCTC-3′, *elavl3* sense: 5′-AATGGTTACTATAATTAG-CACCATG-3′ and antisense: 5′-GAAGGCCTAGTCACTATTGCTCTTT-3′, *myelin protein zero* (*mpz*) sense: 5′-ACGTATACTGACCTGCGGGGAGAT-3′ and antisense: 5′-TGAAAGTAGAAAAATGACCAGAAA-3′.

Microscopy and data analysis

For conventional light microscopy, we used an Axioplan 2 microscope (Zeiss) with Plan-Neofluar 20× (NA0.5) and Plan-Neofluar 40× (NA0.75) objectives, and a BX50 microscope (Olympus) with UPlanApo 20× (NA0.70), UPlanSApo 40× (NA0.95), and UPlanFLN 60× (NA0.90) objectives. For laser scanning confocal microscopy, we used an LSM510 Meta microscope (Zeiss) equipped with Axioplan 2, with Plan-Apochromat 20× (NA0.75) and Plan-Neofluar 40× (NA0.75) objectives, and a C-Apochromat 63× (NA1.2) water-immersion objective, and an FV1000 microscope (Olympus) equipped with BX61 (Olympus) with UPlanSApo 20× (NA0.75) and UPlanSAPO 40× (NA0.90) objectives, and a UPlanSAPO 60× (NA1.35) oil-immersion objective. Images were processed using Adobe Photoshop and Adobe Illustrator.

Anatomical nomenclature

Anatomical nomenclature and abbreviations were used in accordance with Wullimann et al. (1996).

Results

Majority of proliferating cells are distributed in the caudal part of the PGZ in the adult zebrafish optic tectum

Previous studies have shown that in the adult zebrafish optic tectum, BrdU-labeled proliferating cells are equally distributed in the dorsal, caudal, and ventral margins throughout the rostrocaudal extent, except for the very caudal end of the PGZ, which has densely-labeled clusters (Grandel et al., 2006; Marcus et al., 1999; Zupanc et al., 2005). However, detailed quantitative data of proliferating cells within the adult zebrafish optic tectum have not yet been reported. Therefore, we performed a quantitative analysis of the distribution of proliferating cells in the optic tectum of adult zebrafish (Fig. 1). We found that the majority of BrdU-positive cells were located in the caudal, dorsomedial and ventrolateral margins of the PGZ (Figs. 1A–C, arrows, and E); some BrdU-positive cells were sparsely distributed in the non-marginal area of the PGZ and tectum opticum (TeO) (data not shown). In the PGZ region, a total of 775 BrdU-positive cells were observed, and in the rostrocaudal axis, 80% of the rostral region had a relatively small number of BrdU-positive cells (4.0 cells per section) (Fig. 1D, Table 1). However, 20% of the caudal region had a large cluster of BrdU-positive cells (11.7 cells per section) (Fig. 1D, Table 1). In the dorsoventral axis, 92.6% of cells (718 cells) resided in the dorsomedial margin, and only 7.4% of cells (57 cells) resided in the ventrolateral margin (Fig. 1E). These results suggest that the most actively proliferating area of the adult zebrafish optic tectum is the dorsomedial area of the caudal part of the PGZ. Therefore, in the following studies, we focused on the molecular properties of this area.

We also checked the labeling efficiency of proliferating cells with the BrdU and IdU double-labeling method (Burns and Kuan, 2005) (Fig. 1F). Adult fishes were injected with IdU and incubated in IdU solution for 48 or 66 h; BrdU was then introduced in the same manner, followed by incubation for 24 or 6 h, respectively (Fig. 1Fi). Total labeling time for both samples was 72 h. We found that the

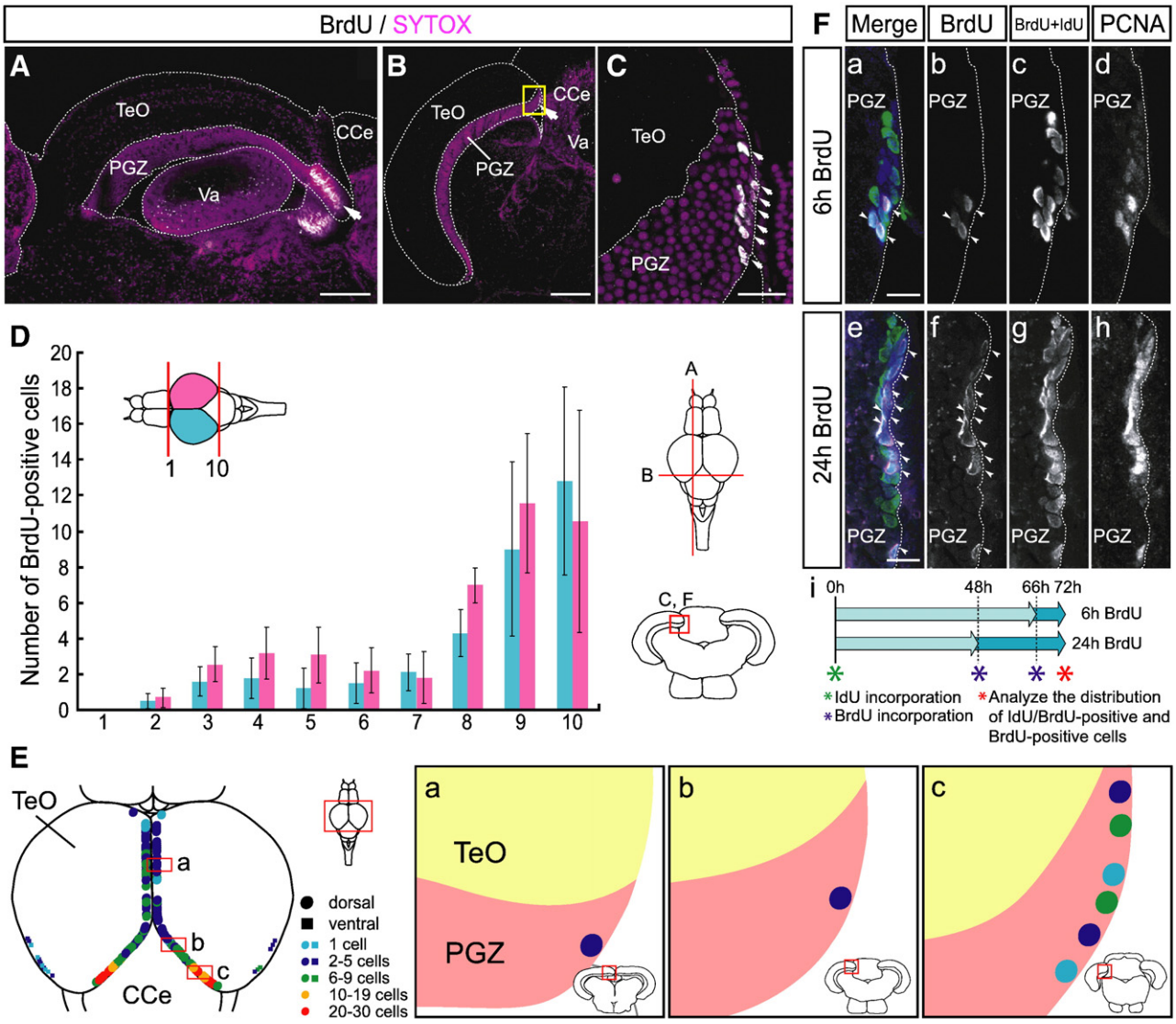


Fig. 1. Proliferating cells are distributed through the dorsomedial area of the caudal region of the PGZ in the adult zebrafish optic tectum. (A–C) Proliferating cells in the adult zebrafish optic tectum. Proliferating cells are labeled by 72-hour BrdU administration (white). Cell Nuclei are stained by SYTOX orange (red). (A) Sagittal section of adult zebrafish optic tectum (single plane, anterior left). A large cluster of BrdU-positive proliferating cells are distributed in the caudal region of the PGZ (arrow). (B) Transverse section of the caudal region of the adult zebrafish optic tectum (single plane, dorsal top). BrdU-positive proliferating cells are distributed through the dorsomedial area of the PGZ (arrow). (C) A high magnification view of the dorsomedial area of the PGZ, indicated by the yellow box in B (single plane, dorsal top). BrdU-positive proliferating cells are located on the dorsomedial margin (arrows). (D) Quantitative data of the distribution of BrdU-positive proliferating cells in the PGZ of the adult zebrafish optic tectum. The whole tectal region is divided into 10 parts along the rostrocaudal axis. Large numbers of proliferating cells are distributed through the caudal region of the optic tectum PGZ. Data are expressed as means ± SEM; n = 3. (E) Schematic drawing of the distribution of proliferating cells in the adult zebrafish optic tectum (dorsal view, anterior top, (a–c) transverse view, dorsal top). Proliferating cells are located along the dorsomedial and ventrolateral margins of the PGZ. The majority of BrdU-positive proliferating cells reside in the dorsomedial area of the PGZ caudal region (c). (F) Dorsomedial distribution of proliferating cells after 6 h (a–d) and 24 h of (e–h) BrdU labeling. One-third and one-half of the proliferating cells are labeled by 6 h and 24 h of BrdU administration, respectively (a, b, e, f, arrowheads). Most of the PCNA-positive cells are labeled by 72 h of continuous administration of IdU or BrdU, and visualized by BrdU antibody which detects both BrdU and IdU (c, g). Whole population of proliferating cells as visualized by immunohistochemistry with anti-PCNA antibody (d, h). IdU and BrdU double-labeling scheme is illustrated in panel i. CCe, corpus cerebelli; PGZ, periventricular gray zone; Tel, telencephalon; TeO, tectum opticum; Va, valvula cerebelli. Scale bars: 200 μm in A, B; 20 μm in C; 10 μm in F.

proliferating cells in the PGZ were partially labeled by 6 or 24 h of BrdU labeling (Figs. 1Fb and Ff), and most of the PCNA-positive proliferating cells were labeled after 72 h with both BrdU and IdU (Figs. 1Fc, Fd, Fg and Fh). Therefore, in subsequent studies (except for Figs. 6A–H), we labeled proliferating cells in the PGZ by BrdU incubation for 72 h.

Table 1
Distribution of BrdU-positive cells in the PGZ of the adult zebrafish optic tectum.

Sections[a]	1	2	3	4	5	6	7	8	9	10
Right PGZ	0	0.7 ± 0.53	2.5 ± 0.95	3.2 ± 1.43	3.1 ± 1.55	2.2 ± 1.29	1.8 ± 1.47	7.0 ± 1.00	11.6 ± 3.89	10.6 ± 6.24
Left PGZ	0	0.5 ± 0.42	1.6 ± 0.79	1.8 ± 1.13	1.2 ± 1.15	1.5 ± 1.16	2.1 ± 1.01	4.3 ± 1.33	9.0 ± 4.87	12.8 ± 5.26

[a] 10-μm-thick serial coronal sections of all the tectal area are divided into 10 groups along the rostrocaudal axis and calculated the mean cell number of each group. Means are expressed ± SEM.

Proliferating cells in the mitotic region of the PGZ express several neural stem/progenitor cell markers

Immunohistochemistry was used to study the expression of neural stem/progenitor cell markers in BrdU-positive proliferating cells located in the dorsomedial area of the caudal region of the PGZ was assessed (Fig. 2). These BrdU-positive cells expressed the proliferation marker PCNA (Figs. 2A–D); most of the PCNA-positive cells were labeled by 72-hour incubation with BrdU. Outside of the PGZ dorsomedial area, PCNA expression was rarely observed (data not shown). Therefore, we designated this PCNA or BrdU-positive area as the mitotic region of the PGZ. Sox2 and Msi1, known to play an important role in the self-renewal of neural precursors in the adult mammalian brain, were also expressed in the mitotic region (Kaneko et al., 2000; Wegner and Stolt, 2005) (Figs. 2E–L, arrowheads). These data suggest that the BrdU-positive cells in the PGZ mitotic region could be neural progenitor cells.

Post-mitotic cells in the ventral edge of the PGZ possess radial glial properties

Interestingly, we found that neural stem/progenitor marker Msi1 and Sox2 were also observed in the ventral edge of the PGZ (Figs. 2E–L, yellow arrows). To address the correct localization of these cells, we examined the distribution of neuronal and glial cells in the PGZ using Tg (*elavl3*:GFP) strains which label the majority of neuronal cells with GFP under the control of the *elavl3* promoter (Park et al., 2000), and Tg (*gfap*:GFP) strains, which can label *gfap*-positive glial cells with GFP under the control of the *gfap* promoter (Bernardos and Raymond, 2006) (Figs. 3A–D). We also examined the immunostaining of several glial and neural progenitor markers such as GFAP, S100β, Fabp7a, and Sox2 (Figs. 3E–T). In Tg (*elavl3*:GFP) strains, only a few cells expressed a strong *elavl3*:GFP signal and extended a dendrite-like processes toward upper layer structures (Figs. 3A and B, arrowheads); however, the majority of cells showed a weak expression of *elavl3*:GFP and were

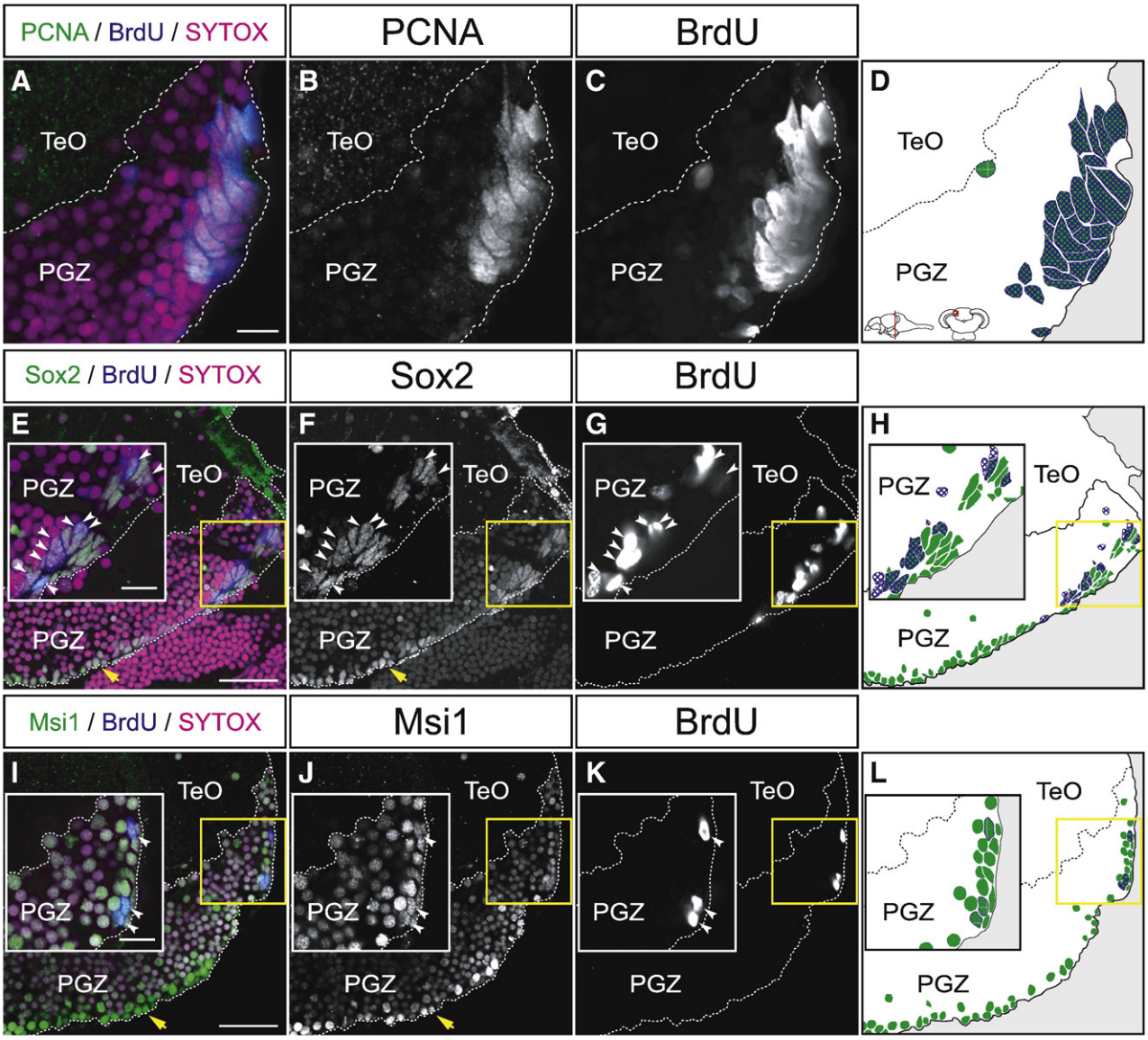


Fig. 2. Proliferating cells in the dorsomedial area of the PGZ express neural stem/progenitor cell markers. (A–L) Expression of PCNA (A–D), Sox2 (E–H), and Msi1 (I–L) in the dorsomedial area of the PGZ of the adult zebrafish optic tectum (60 μm transverse sections, single planes, dorsal top). Proliferating cells are labeled with BrdU after 72 h of incubation. Insets in E–L show magnified views of the yellow-boxed areas. (A–D) Most PCNA-positive cells incorporate BrdU after 72 h of BrdU administration. (E–F) The majority of BrdU-positive proliferating cells (insets, arrowheads), and the cells that reside in the ventral edge of the PGZ (yellow arrows) express Sox2. (I–L) A subset of BrdU-positive cells (insets, arrowheads), and the cells that reside in the ventral edge of the PGZ (yellow arrows) express Msi1. CCe, corpus cerebelli; PGZ, periventricular gray zone; TeO, tectum opticum. Scale bars: 10 μm in A, insets of E, I; 30 μm in E, I.

abundant in the PGZ, except for the mitotic region and the cells in the ventral edge of the PGZ, which specifically expressed S100β (Figs. 3A and B).

In contrast, in Tg (*gfap*:GFP) strains, *gfap*:GFP-positive glial cells were specifically observed in the ventral edge of the PGZ (Figs. 3C and D). Remarkably, these cells produced thin layer structure and extended radial fibers to the surface of the optic tectum (Figs. 3C and D, arrowheads); all *gfap*:GFP-positive cells expressed GFAP, S100β, Fabp7a, and Sox2 (Figs. 3E–T). According to these results, we determined that the cells in the ventral edge of the PGZ possess radial glial properties. We designated these *gfap*:GFP-positive radial glial populations and *elavl3*:GFP-positive neuronal populations as the deep and superficial layers of the PGZ, respectively.

Proliferating cells in the mitotic region of the PGZ does not have glial properties but have neuroepithelial characteristics

In the adult mammalian brain, neural stem/progenitor cells show astroglial or radial glial characteristics (Doetsch, 2003). Therefore, we examined whether these cells expressed *gfap*:GFP and S100β, which are usually expressed in astrocytes and radial glia, and Fabp7a, which is expressed in radial glia (Fig. 4) (Götz and Barde, 2005; Hartfuss et al., 2001; Liu et al., 2003; Wainwright et al., 2004). In the mitotic region, *gfap*:GFP, and S100β were not expressed (Figs. 4A–D and I–L), while expression of *fabp7a* was observed (Figs. 6E–H), however we could not detect Fabp7a-positive cells in this region by immunohistochemistry (Figs. 4E–H). These results suggest that the neural stem/progenitor cells residing in the mitotic region do not have glial characteristics.

In the adult zebrafish cerebellum, neural stem cells show not glial but neuroepithelial characteristics (Kaslin et al., 2009). These stem cells maintain ventricular contact and apical–basal polarity. According to these observations, we examined the localization of apical markers such as ZO-1, γ-tubulin and aPKC in the mitotic region of the PGZ (Fig. 5). We found polarized distribution of apical markers in the most medial PCNA-positive cells which face the ventricle (Figs. 5A, B, E, F, I and J, arrow heads). These results suggest that these cells have neuroepithelial characteristics.

Progenitor cells in the mitotic region of the PGZ contribute to both neuronal and glial cell lineages

To investigate whether the proliferating cells in the mitotic region of the PGZ contribute to both neuronal and glial cell lineages, we performed BrdU pulse label analysis (Fig. 6). In each stage, we examined the distribution of BrdU-positive cells along with fluorescent *in situ* hybridization by using *elavl3* and *fabp7a* as markers for neuronal cells in the superficial layer and glial cells in the deep layer of the PGZ, respectively (Figs. 6A–H and U). We found that the majority of BrdU-positive cells showed a strong *elavl3* expression at 2 weeks post-BrdU administration (Figs. 6B and I–L, arrowheads), and then maintained weak *elavl3* expression till at least 2 months post-BrdU administration, suggesting that these cells differentiated into neuronal cells in the superficial layer of the PGZ. We also found that some BrdU-positive cells were *elavl3*-negative but *fabp7a*-positive in the ventral edge of the PGZ (Figs. 6C, D, G and H, arrowheads). To confirm this observation, we examined BrdU incorporation in the *gfap*–GFP-positive cells at 2 weeks post-BrdU administration (Figs. 6M–P). We found that some *gfap*–GFP-positive cells, located close to the mitotic region, incorporated BrdU, suggesting that these BrdU-positive cells differentiated into glial cells in the deep layer of the PGZ (Figs. 6M–P, arrowhead). These results suggest that the progenitor cells in the mitotic region contributed to both neuronal and glial cell lineages (Figs. 6V and W). To address the existence of self-renewing stem cells, we examined the distribution of BrdU-positive cells by immunostaining with the proliferating cell marker pH3, which is phosphorylated in the M-phase, at 1 month post-BrdU administration (Figs. 6Q–T). We found that some BrdU-positive cells still remained in the most medial part of the PCNA-positive region and showed pH3 immunoreactivity even though most of the BrdU-positive cells were localized in the pH3-negative post-mitotic region (Figs. 6R and S). These results suggest the existence of self-renewing stem cells in the mitotic region of the PGZ (Fig. 7).

To determine the final differentiation status of BrdU-positive cells, we examined the expression of several neurotransmitter markers (Figs. 7A–P) 1 month after BrdU administration. Some BrdU-positive cells expressed glutamatergic neuronal markers *slc17a6a* (also called *vglut2.2*) and *slc17a6b* (also called *vglut2.1*) (Higashijima et al., 2004) (Figs. 7A–H, arrowheads), and GABAergic neuronal markers *gad1* and *gad2* (Martin et al., 1998) (Figs. 7I–P, arrowheads). We also examined the expression of the immunoglobulin superfamily molecule, *myelin protein zero* (*mpz* also called *P0*) (Schweitzer et al., 2003) (Figs. 7Q–T). In zebrafish, *mpz* is expressed in CNS oligodendrocytes (Schweitzer et al., 2003; Yoshida and Macklin, 2005), while *mpz* expression in the mammalian PNS is restricted to Schwann cells (Spiryda, 1998). We found that some BrdU-positive cells express *mpz* (Figs. 7Q and T, arrowheads). Alongside the results shown in Fig. 6, these results demonstrate that the proliferating cells in the mitotic region of the PGZ differentiate into multiple cell lineages.

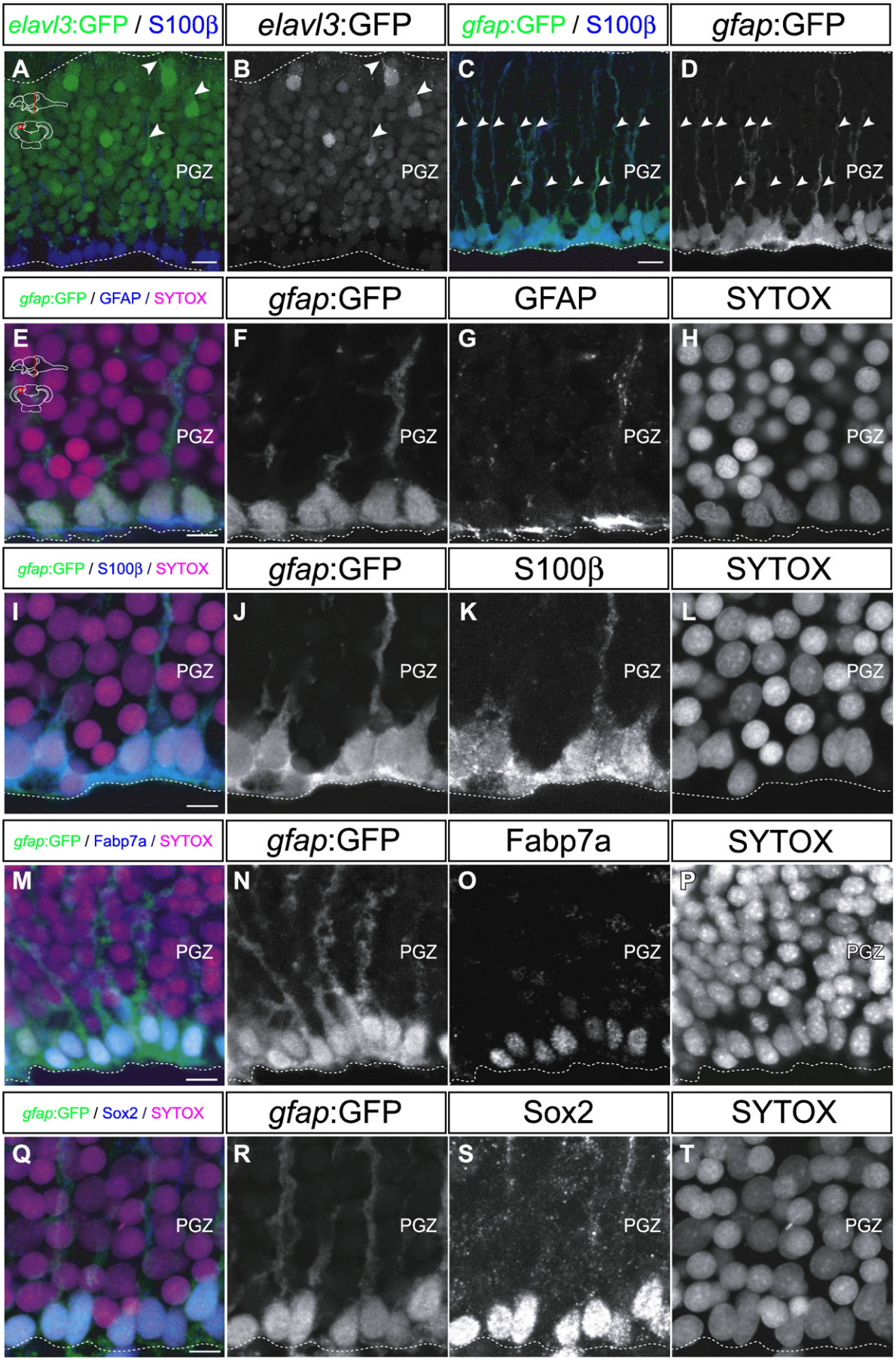
Discussion

In this study, we characterized neural stem/progenitor cells in the adult zebrafish optic tectum. Proliferating cells, located in the dorsomedial area of the caudal part of the PGZ, expressed several neural stem/progenitor cell markers. These proliferating cells generated both neuronal and glial cell lineages in the PGZ (Fig. 8). In addition, some of these cells retained their self-renewal capacity in the most medial part of the mitotic region, suggesting the existence of neural stem cells. Interestingly, these neural stem/progenitor cells did not express any astrocyte/radial glial cell markers, which are usually expressed in neural stem/progenitor cells in adult mammalian brains but show neuroepithelial characteristics. These cells differentiate into multiple lineages. We also found that the radial glial cells in the deep layer of the PGZ retained the expression of neural stem/progenitor cell markers, which were observed in the proliferating cells of the mitotic region, suggesting the possibility that these glial cells also have the potential to be neural progenitors.

Active proliferating region of optic tectum in adult brain

In this study, we quantitatively analyzed the distribution of proliferating cells within the optic tectum of adult zebrafish. Previously, several studies had reached different conclusions regarding the distribution of proliferating cells in this region.

Zupanc et al. (2005) reported that at 2 h post-BrdU administration, most of the BrdU-labeled cells were equally distributed throughout the whole tectal region in the rostrocaudal extent, except for the caudal end of the PGZ. However, Grandel et al. (2006) showed that the tectal proliferating zone was only observed in the medial margin of the PGZ. In the present study, we found that the majority of BrdU-labeled cells were located in the dorsomedial area of the PGZ, in agreement with the finding of Grandel et al. In the rostrocaudal axis, quantitative analysis demonstrated that 20% of the caudal region of the dorsomedial area of the PGZ had a large cluster of BrdU-labeled cells. According to these results, we concluded that the majority of neuronal and glial cells are generated from this actively proliferating region of the optic tectum in the adult zebrafish brain.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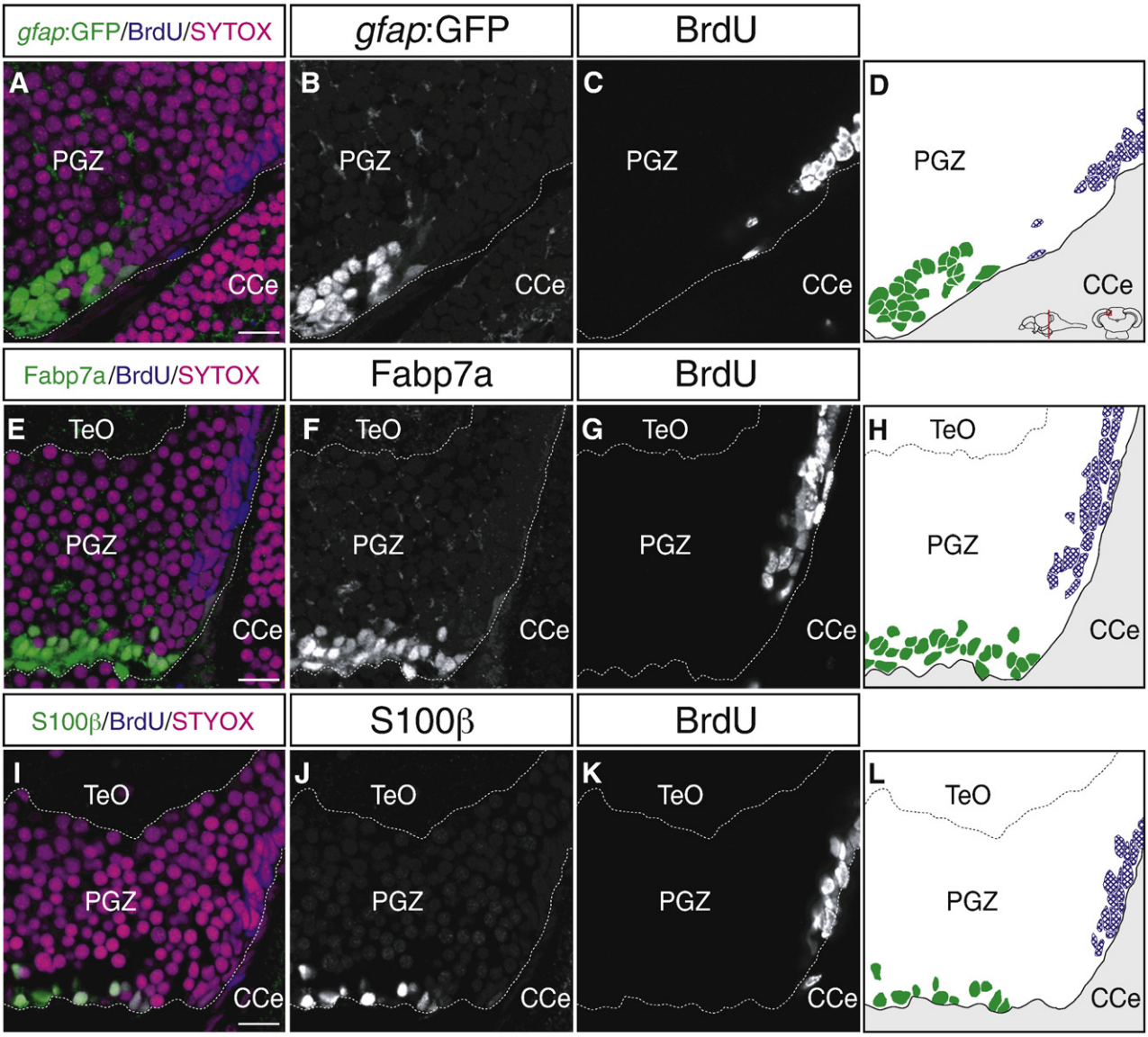


Fig. 4. Proliferating cells in the PGZ do not express glial markers. (A–L) Expression of *gfap*:GFP (A–D), Fabp7a (E–H), and S100β (I–L) in the PGZ of the adult zebrafish optic tectum (60 μm transverse sections, single planes, dorsal top). Proliferating cells are labeled after 72 h of BrdU administration. In the medial region of the PGZ, BrdU-positive proliferating cells (blue) do not express the glial markers, *gfap*:GFP, Fabp7a and S100β (green); these glial markers are expressed in the deep layer cells. CCe, corpus cerebelli; PGZ, periventricular gray zone; TeO, tectum opticum. Scale bars: 10 μm.

Neural stem/progenitor cells in the mitotic region of adult zebrafish optic tectum maintain neuroepithelial characteristics

In this study, we revealed the characteristics of proliferating cells in the PGZ of the optic tectum. These cells expressed several neural stem/progenitor cell markers such as *sox2* and *msi1* generated neuronal and glial cell lineages, and were self-renewing. These properties are in accordance with the standard definition of neural stem/progenitor cells. In addition to these characteristics, canonical neural stem/progenitor cells in the adult brain possess glial identities (Doetsch, 2003). In the mammalian telencephalon, neural stem cells in the subventricular zone of the lateral ventricles and the subgranular zone in the hippocampus possess astroglial properties; in the adult zebrafish brain, ventricular telencephalic progenitors also possess glial molecular characteristics and form a rostral migratory stream towards the olfactory bulb, similar to that observed in the subventricular zone of the adult mammalian brain (Adolf et al., 2006). In the midbrain, *her5*:GFP-positive neural stem cells lining the midbrain–hindbrain boundary express the glial marker GFAP (Chapouton et al., 2006; Doetsch, 2003). Surprisingly, we found that the neural stem/progenitor cells in the mitotic region of the PGZ did not express glial markers, and some of them which face the ventricle were highly polarized. A similar type of neural stem/progenitor cells is the neuroepithelial stem cells in the ventricular zone of the developing

Fig. 3. Cells constitute the deep layer of the PGZ are radial glia. (A–D) Distribution of *elavl3*:GFP-positive cells (A, B) and *gfap*:GFP-positive cells (C, D) in the PGZ of the adult zebrafish optic tectum (60 μm transverse sections, dorsal top). (A, B) The *elavl3*:GFP-positive cells are distributed through a broad area of the PGZ, except for the S100β-positive ventral edge. Only a few cells express strong GFP signal and extend dendrite-like processes toward the surface layers of the optic tectum (arrowheads). (C, D) The *gfap*:GFP-positive cells are distributed along the ventral edge of the PGZ, and extend radial fibers (arrowheads). (E–T) Magnified views of the *gfap*:GFP-positive cells along the ventral edge of the PGZ (60 μm transverse section, dorsal top). The *gfap*:GFP-positive cells constitute the deep layer of the PGZ. These cells show immunoreactivities with glial markers, such as GFAP, S100β and Fabp7a (E–P), and the neural stem/progenitor marker Sox2 (Q–T). Scale bars: 10 μm in A, C; 5 μm in E, I, M, Q.

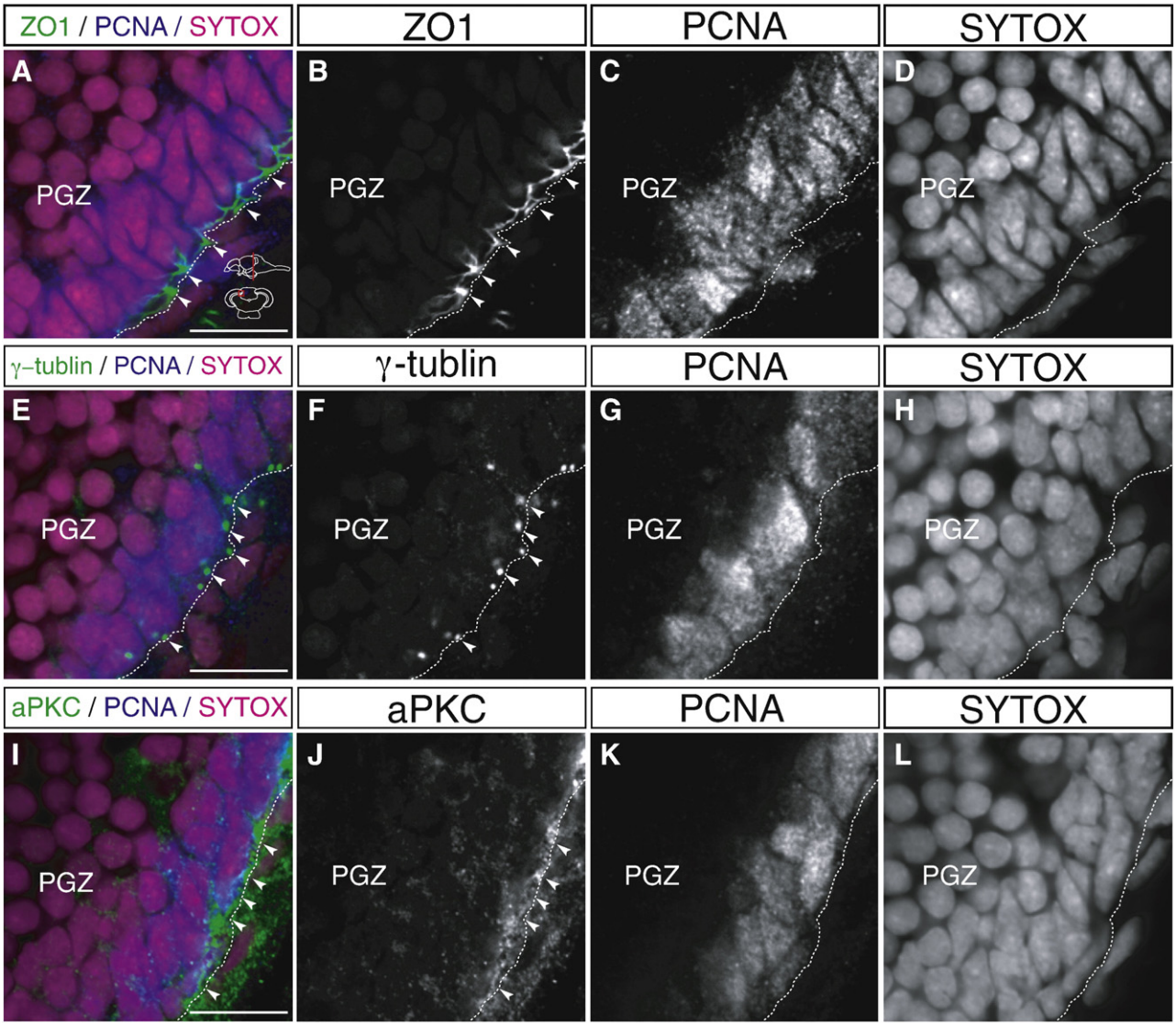


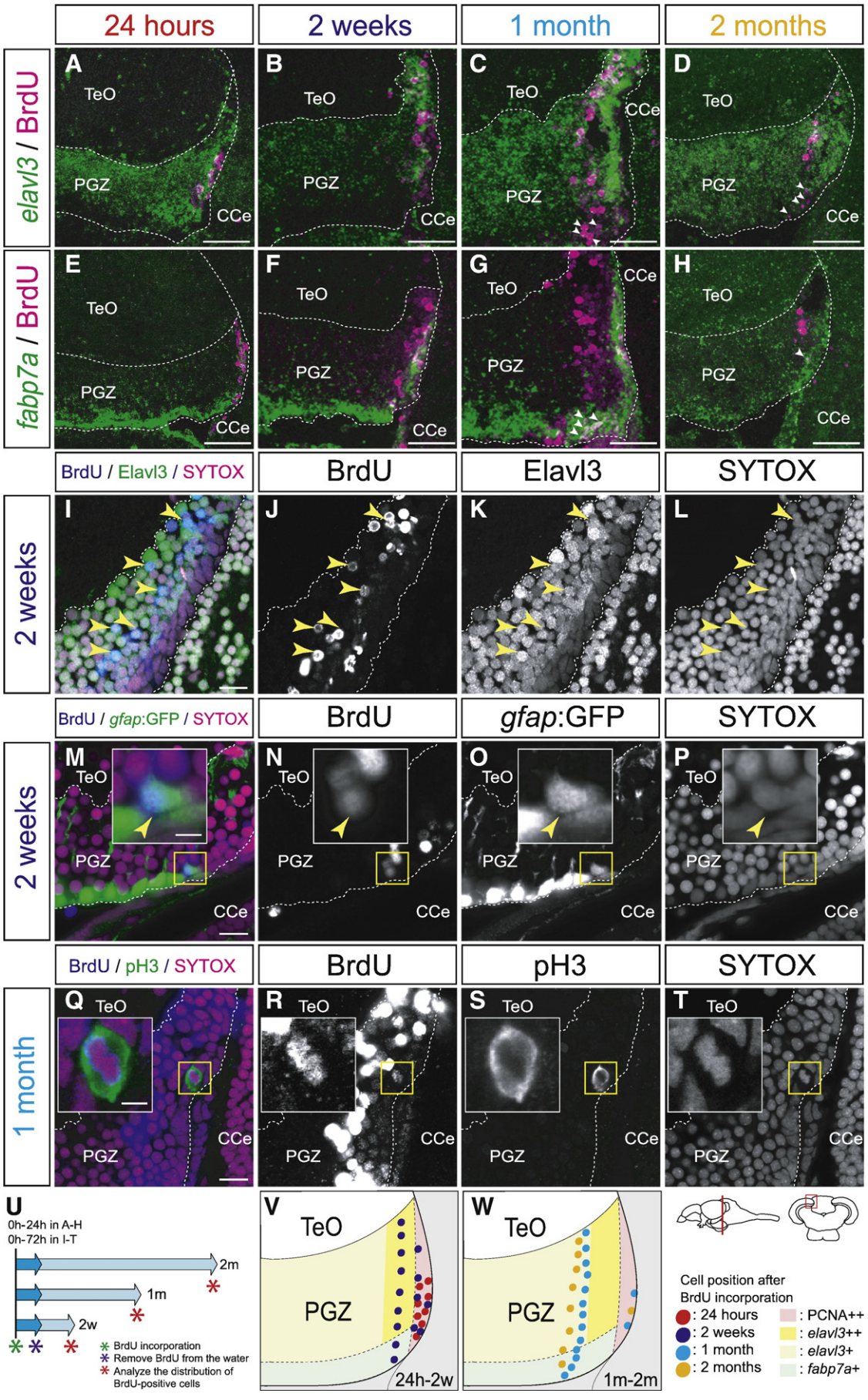
Fig. 5. Proliferating cells which face the ventricle maintain apical–basal polarity. (A–L) Localization of apical markers, ZO1 (A–D), γ-tubulin (E–H), and aPKC (I–L) in the proliferating cells of the PGZ of the adult zebrafish optic tectum (60 μm transverse sections, single planes, dorsal top). The proliferating cells are visualized by immunohistochemistry with anti-PCNA antibody. The proliferating cells which localize near the ventricle show highly polarized expression of apical markers such as ZO1 (A, B, arrowheads), γ-tubulin (E, F, arrowheads), and aPKC (I, J, arrowheads). Scale bars: 10 μm.

vertebrate brain. Neuroepithelial cells are apical–basal polarized and contact both the apical (ventricular) and basal (pial) surfaces (Merkle and Alvarez-Buylla, 2006). Recently, Kaslin et al. (2009) reported that cerebellar stem cells in adult zebrafish did not possess radial glial properties but instead displayed neuroepithelial properties. These cells maintained their apical–basal polarity and ventricular contact throughout the embryonic to adult stages. These findings, including ours, suggest that the adult teleost brain has 2 types of neural/progenitor cells—one has canonical glial properties similar to mammalian neural progenitors, while the other has non-radial glial properties and is unique to the adult teleost brain.

Radial glial cells in the deep layer of the PGZ express several neural stem cell markers

Our study revealed that the radial glial cells in the deep layer of the PGZ continued to express several neural stem cell markers such as *sox2* and *msi1* which are also expressed by proliferating cells in the

Fig. 6. Neural stem/progenitor cells in the mitotic region of the PGZ generate both neuronal and glial cell lineages. (A–H) Distributions of BrdU-positive cells in the *elavl3*-positive superficial layer (A–D), and the *fabp7a*-positive deep layer (E–H) of the PGZ in the adult zebrafish optic tectum at 24 h (A, E), 2 weeks (B, F), 1 month (C, G), and 2 months (D, H) post-BrdU administration (14 μm transverse sections, stacked images, dorsal top). After 2 weeks post-BrdU administration, most of the BrdU-positive cells express *elavl3* (B–D), however, some BrdU-positive cells express *fabp7a* but not *elavl3* (C, D, G, H, arrowheads). (I–P) Expression of Elavl3 (I–L) and *gfap*:GFP (M–P) in the BrdU-positive cells at 2 weeks post-BrdU administration (60 μm transverse sections, single planes, dorsal top). Insets in M–P show magnified views of the yellow-boxed areas. The majority of BrdU-positive cells expressed the neuronal marker Elavl3 (I–L), and some BrdU-positive cells expressed the glial maker *gfap*:GFP (M–P, insets, arrowheads). (Q–T) Distribution of pH3-positive cells in the PGZ at 1 month post-BrdU administration (60 μm transverse sections, single planes, dorsal top). Insets in Q–T show magnified views of the yellow-boxed areas. At 1 month post-BrdU administration, majority of BrdU-positive cells leave the dorsomedial margin (Q, R), while BrdU-positive cells facing the ventricle still undergo cell division and expresses the M-phase marker, pH3 (Q–T, insets). (U) BrdU pulse labeling scheme. (V, W) Summaries of the distribution of BrdU-positive cells at 24 h to 2 weeks (V), and at 1 month to 2 months (W) post-BrdU administration. CCe, corpus cerebelli; PGZ, periventricular gray zone; TeO, tectum opticum. Scale bars: 50 μm in A–H; 10 μm in I, M, Q; 3 μm in inset of M, Q.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BrdU incorporation
Remove BrdU from the water
Analyze the distribution of BrdU-positive cells
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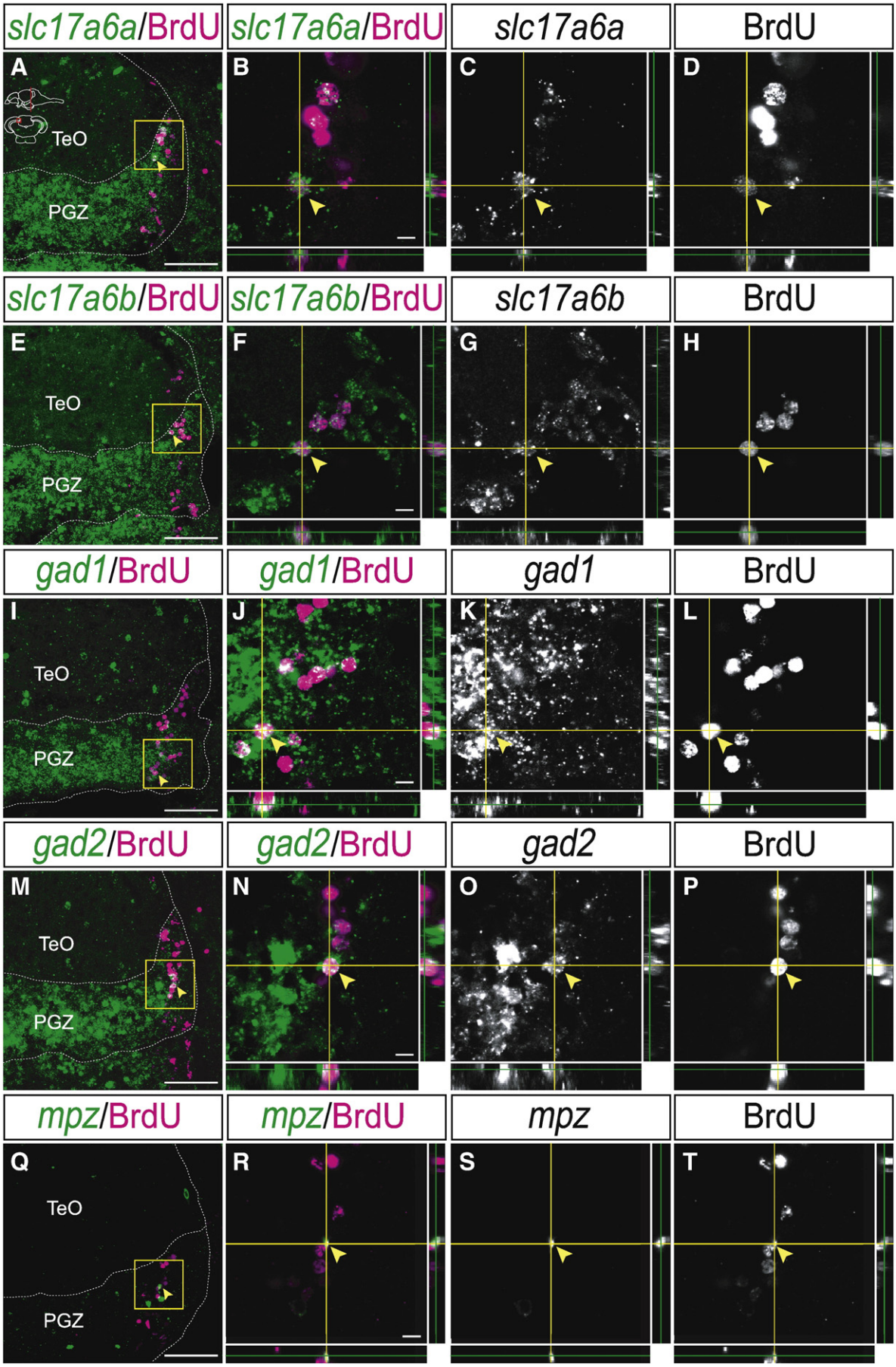
: fabp7a+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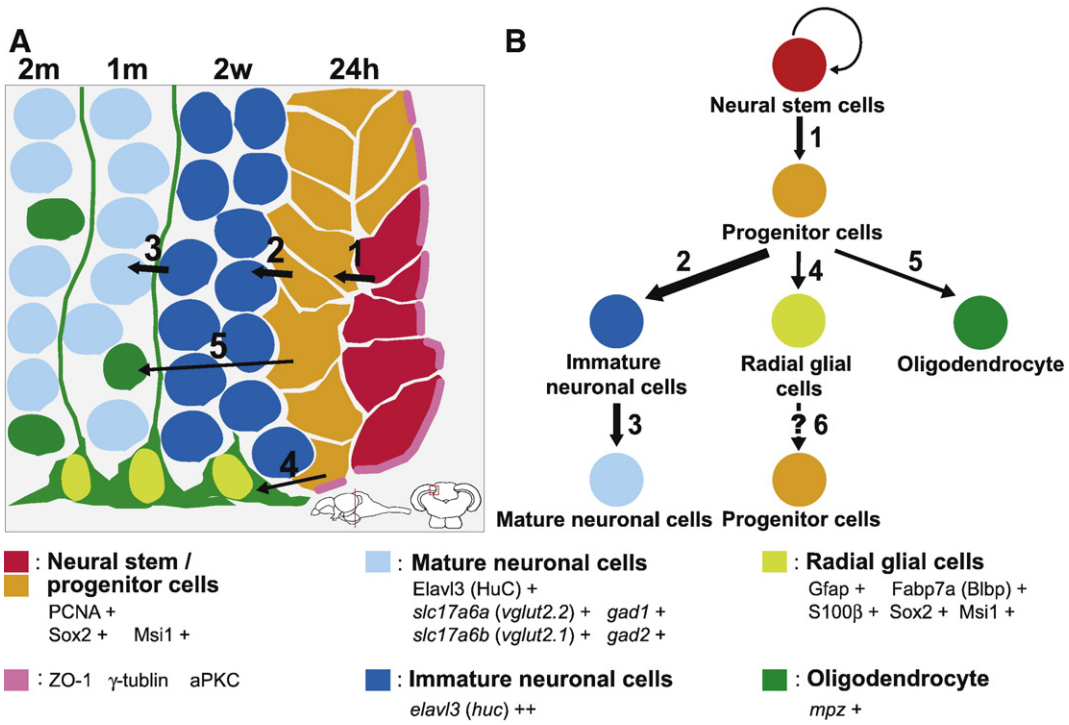

Fig. 8. Schematic drawings of the cell lineage of neural stem/progenitor cells in the PGZ of the adult zebrafish optic tectum. (A) Distribution of neural stem cells (red), progenitor cells (orange), immature neuronal cells (dark blue), mature neuronal cells (light blue), radial glial cells (light green), and oligodendrocyte (dark green) in the PGZ of the adult zebrafish optic tectum. (B) Cell lineage of neural stem/progenitor cells in the adult zebrafish optic tectum. 1: The neural stem cells in the mitotic region of the PGZ facing to the ventricle generate progenitor cells. 2: Most progenitor cells differentiate into immature neuronal cells, and show strong *elavl3* expression around 2 weeks after final cell division. 3: Strong expression of *elavl3* in immature neuronal cells is reduced, and they differentiate into mature glutamatergic or GABAergic neurons. 4: Some progenitor cells differentiate into radial glial cells at least 2 weeks after final cell division. 5: A small portion of progenitor cells differentiate into oligodendrocytes. 6: Radial glial cells maintain expression of neural stem/progenitor cell markers; therefore, we assume that these cells possess the potential of progenitor cells.

mitotic region of the PGZ. These properties partially fulfill the definition for canonical neural stem/progenitor cells except for their self-renewing capacity and the generation of multiple cell types such as neurons and glia. We did not observe BrdU incorporation in these radial glial cells after at least 24-hour BrdU labeling. However, we found that a few cells showed PCNA immunoreactivity (data not shown), suggesting the possibility that some of these cells are long-lasting proliferating cells. So far, several studies have reported the existence of radial glia in the deep layer of the PGZ in adult teleosts (Arochena et al., 2004; Kalman, 1998; Kinoshita et al., 2005; Stevenson and Yoon, 1981; Stevenson and Yoon, 1982). These radial glial cells showed intermediate filaments vimentin or GFAP immunoreactivities (Arochena et al., 2004; Kalman, 1998). Interestingly, Stevenson and Yoon (1978, 1980, 1981, 1982) reported the existence of mitotic radial glia-like cells (periependymal (PE) cells) in the PGZ of the adult goldfish optic tectum. In their study, the mitotic activity of PE cells was enhanced by regeneration of the optic nerve. A similar correlation between tectal cell proliferation and optic nerve input to the optic tectum during retinal fiber regeneration was also reported by Raymond et al. (1983). These studies, including ours, imply the potential of radial glial cells in the adult teleost optic tectum to function as neural stem/progenitor cells.

We hypothesize that there are 2 types of neural stem/progenitor cells in the optic tectum of the adult teleost—fast-proliferating cells in the marginal area of the PGZ, which possess non-glial properties, and slow-proliferating cells in the deep layer of the PGZ, which possess canonical radial glial properties. The fast-proliferating cells produce large numbers of neuronal and glial cells for the continuous growth of the optic tectum. The slow-proliferating cells maintain the already established structure of the optic tectum. Recently, Suh et al. (2007) reported that in mice, the Sox2, GFAP, BLBP, and Musashi1-positive radial glia-like cells in the subgranular zone of the hippocampus proliferated only when the mice were stimulated by voluntary running. We assume that a similar activity-dependent mechanism may also function in the regulation of neural stem/progenitor cells in the optic tectum of the adult teleost.

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Fig. 7. Neural stem/progenitor cells in the mitotic region of the PGZ differentiate into the glutamatergic or GABAergic neurons and oligodendrocytes. (A–P) Expression of glutamatergic and GABAergic neuronal markers in the BrdU-positive cells in the PGZ of the adult zebrafish optic tectum at 1 month post-BrdU administration (14 μm transverse sections, stacked images, dorsal top). (A–H) Some of the BrdU-positive cells express glutamatergic neuronal markers, *slc17a6a* (A–D, arrowheads) and *slc17a6b* (E–H, arrowheads) (B–D: high magnification of yellow-boxed area in A, F–H: high magnification of yellow-boxed area in E). (I–P) Some of the BrdU-positive cells express GABAergic neuronal markers, *gad1* (I–L, arrowheads) and *gad2* (M–P, arrowheads) (J–L: high magnification of yellow-boxed area in I, N–P: high magnification of yellow-boxed area in M). (Q–T) Expression of oligodendrocyte marker, *mpz* in the BrdU-positive cells in the PGZ of the adult zebrafish optic tectum at 1 month post-BrdU administration (arrowheads) (14 μm transverse sections, stacked images, dorsal top) (R–T: high magnification of the yellow-boxed area in Q). CCe, corpus cerebelli; PGZ, periventricular gray zone; TeO, tectum opticum. Scale bars: 50 μm in A, E, I, M, Q; 5 μm in B, F, J, N, R.

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