Developmental Biology 342 (2010) 26–38

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

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ARTICLE INFO

Article history:
Received for publication 26 September 2009
Revised 11 March 2010
Accepted 15 March 2010
Available online 24 March 2010

Keywords:
Adult neurogenesis
Neural stem cells
Optic tectum
Radial glia
Zebrafish

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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doi:10.1016/j.ydbio.2010.03.008

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