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Neural stem cells and neurogenesis in the adult zebrafish brain: Origin, proliferation dynamics, migration and cell fate

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Abstract

Lifelong neurogenesis in vertebrates relies on stem cells producing proliferation zones that contain neuronal precursors with distinct fates. Proliferation zones in the adult zebrafish brain are located in distinct regions along its entire anterior–posterior axis. We show a previously unappreciated degree of conservation of brain proliferation patterns among teleosts, suggestive of a teleost ground plan. Pulse chase labeling of proliferating populations reveals a centrifugal movement of cells away from their places of birth into the surrounding mantle zone. We observe tangential migration of cells born in the ventral telencephalon, but only a minor rostral migratory stream to the olfactory bulb. In contrast, the lateral telencephalic area, a domain considered homologous to the mammalian dentate gyrus, shows production of interneurons and migration as in mammals. After a 46-day chase, newborn highly mobile cells have moved into nuclear areas surrounding the proliferation zones. They often show HuC/D immunoreactivity but importantly also more specific neuronal identities as indicated by immunoreactivity for tyrosine hydroxylase, serotonin and parvalbumin. Application of a second proliferation marker allows us to recognize label-retaining, actively cycling cells that remain in the proliferation zones. The latter population meets two key criteria of neural stem cells: label retention and self renewal.

Keywords: Adult neurogenesis; Proliferation zones; Neural stem cells; *Danio rerio*

Introduction

Vertebrate neurogenesis occurs not only during embryogenesis but also during adult stages (Alvarez-Buylla and Kirn, 1997; Doetsch and Scharff, 2001; Zupanc, 2001). In rodents and songbirds, adult neurogenesis is observed in highly restricted spatial domains that generate new cells destined for distinct telencephalic regions. In rodents, these regions are the granular and peri-glomerular layers of the olfactory bulb that receive new interneurons (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Hack et al., 2005) and the dentate gyrus in the hippocampus where new granule neurons are produced during adulthood (Altman and Das, 1965; Cameron et al., 1993; Kaplan and Bell, 1984; Seri et al., 2001). In songbirds, newborn neurons are added to diverse regions in the telencephalon. The best studied example is the HVC nucleus in canaries where interneurons and long projecting neurons are added throughout life and survive for several months (Kirn et al., 1991; Nottebohm et al., 1994), implying growth of this particular brain nucleus at that time. In contrast, new cells in the hippocampus do not cause brain growth but rather indicate a constitutive, low turnover rate of neurons (Barnea and Nottebohm, 1996). In both vertebrate groups, proliferation zones that generate the new neurons are situated close to the forebrain ventricles. In birds, the ventricular zone directly contacts the forebrain ventricular lumen. In mammals, the subventricular/subependymal zone is located below the ependymal layer lining the forebrain ventricles, while the subgranular zone, a derivative of the subventricular zone, has split off in adults.

While in mammals and birds, constitutive turnover of neurons has been emphasized, net brain growth occurs during adulthood in reptiles, amphibians and fish (reviewed in Font et al., 2001; Kaslin et al., in press). The forebrains of lizards show an age- and bodyweight-dependent increase in neuronal

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numbers. In teleosts, the best studied brain parts are the retina and the optic tectum, which both grow from discrete proliferation zones near the margins of these structures (Johns, 1977; Raymond and Easter, 1983; Marcus et al., 1999).

It has long been known that brains of adult teleost fish show widespread cell proliferation in all subdivisions along the rostrocaudal axis (Kirsche, 1967; Zupanc and Horschke, 1995; Zupanc et al., 2005; Ekström et al., 2001). The generation of new cells may in fact account for the lifelong brain growth observed in different fish species (Brandstätter and Kotrschal, 1990). However, discrete zones of adult proliferation have only recently been described in a complete and detailed manner (Ekström et al., 2001) as this has been done in mammals. The widespread occurrence of specific proliferation zones throughout the brains of adult teleosts immediately suggests adult neurogenesis in these areas. In view of the general importance of adult proliferation and neurogenesis in vertebrates, we systematically investigated whether these processes take place in a model teleost, especially whether neurogenesis is as widespread as proliferation or whether it is likewise restricted to few locations as in amniote brains. We chose the zebrafish *Danio rerio* because of its advantages as a vertebrate model organism and designed experiments to determine origin and fate of newly generated cells in the brains of adults. Recently, Zupanc et al. (2005) independently described proliferation zones and neurogenesis in the brain of adult zebrafish. However, the main focus of this publication is on the cerebellum, where they note considerable proliferative activity but fail to detect widespread neurogenesis. Furthermore, less attention has been given to other brain regions, notably the diencephalon which therefore does not allow an overall evaluation of proliferation zones among teleosts, as described by others (Zupanc and Horschke, 1995; Ekström et al., 2001). Our goal is an experimental analysis of the general mechanisms underlying neurogenesis that apply to all vertebrate classes, and we are thus in favor of a more comprehensive account of this phenomenon.

In the present study, we describe 16 distinct proliferation zones in the entire brain, mostly located along the ventricular surface, but also deeper in the brain parenchyma. They occur in adults from 6 months to at least 2.5 years of age, which is about 75% of the fishes lifetime (Gerhard et al., 2002). We have determined distinct patterns of migration of newly generated cells away from these proliferation zones and followed their differentiation into neurons by immunohistochemistry. Interestingly, new aminergic neurons (tyrosine hydroxylase or serotonin positive) are added in the olfactory bulb and diencephalon of the adult zebrafish brain indicating that new long projecting neurons arise throughout adulthood. The persistence of adult neurogenic proliferation zones suggests their maintenance by a population of stem cells that have the capacity to self-renew, thereby fulfilling one criterion of stemness (Potten and Loeffler, 1990). We have found different types of proliferating cells within the proliferation domains, where many move away from their place of birth during a prolonged time period, while a few cells remain behind and show an actively cycling behavior characteristic of stem cells. Our results identify in the proliferation zones in the brain of adult zebrafish a mobile pool of neuronal precursor cells and a stationary, label-retaining population that we suggest to represent the neural stem cell pool.

Materials and methods

Experimental animals used throughout this study were all adult fish from the *gol-b1* line in the AB genetic background (Streisinger et al., 1981; Brand et al., 2002). At 6 months, zebrafish are well into adulthood since zebrafish are sexually mature at 3 months.

Bromo- and Iododesoxyuridine (BrdU/IdU) treatments

We injected 1 μl/100 mg body weight of a 10 mM BrdU solution intraperitoneally into eight 6-month-old adult zebrafish followed by an overnight (ON) survival time after which the fish were anesthetized with MS222 until the opercular movement had ceased. The brains were then exposed or removed in ice-cold E3 and fixed in 2% Paraformaldehyde in 0.1 M phosphate buffer (PFA) overnight (ON). We prepared whole mount preparations which we immunostained with an anti-BrdU antibody (see below; Figs. 2O, P). To increase the pulse time, we incubated fish in a 1 mM or 10 mM BrdU solution in E3, titrated to pH 7.5. Ten millimolars BrdU gave more consistent labeling results. We incubated fish, 27 mm–29 mm in length, for 2 h (1 fish), 4 h (1 fish), 8 h (3 fish), 16 h (1 fish), 24 h (2 fish) or 48 h (15 fish). Fish incubated in BrdU for 2 h–24 h were killed after the incubation, serially cryosectioned and stained to determine proliferation zones in the adult brain. The brain shown in Fig. 2Q was taken from a fish, incubated 24 h in 10 mM BrdU solution, halved and fluorescently stained as a whole mount. For pulse chase experiments, fish were incubated in 10 mM IdU (Burns and Kuan, 2005) or BrdU for 48 h and allowed to survive in aquaria containing regular fish water for 5 days (d), 12d or 46d after which they were killed and fixed in PFA as above. To determine the location of labeled cells with respect to the proliferation zones at the time of fixation, BrdU, IdU and PCNA (see below) were used as second proliferation markers (see Results).

Histology

All solutions were prepared in 0.1 M phosphate buffer. Fixed heads were decalcified and infiltrated in 20% EDTA, 10% sucrose ON and in 20% EDTA, 20% sucrose ON. They were then embedded in 7.5% gelatine 20% sucrose and frozen. Fourteen-micrometer-thick cryosections were prepared on a Microm HM 560 cryostat.

Immunohistochemistry

Sections were dried for 0.5 h at room temperature (RT), and gelatine was removed in PBS at 37°C. After washing in 0.3% PBX (0.1 M phosphate buffer, 0.3% Triton-X100), sections were incubated with the primary antibody ON at 4°C: BrdU, monoclonal mouse (Mab), Roche, 1:500; BrdU and IdU, Mab Becton Dickinson, 1:500; BrdU only, monoclonal rat, Serotec, 1:500; HuC/D, Mab, 1:50; Molecular Probes, PCNA, Mab Dako, 1:500; tyrosine hydroxylase, Mab Immunostar, 1:1000; parvalbumin, Mab Chemicon, 1:1000; serotonin, polyclonal rabbit, Sigma, 1:4000; S100β, polyclonal rabbit, Dako, 1:500. After 3 washes in PBX, sections were incubated with species-specific secondary antibodies coupled to Alexa Fluor 488 or 546 (Molecular Probes) or with biotinylated horse anti-mouse supplied by the Vectastain Elite Kit, Vector laboratories, PK-6103 for 1 h and 0.5 h at RT, respectively, washed 3× in PBX and embedded in 50% Glycerol in PBS or visualized by the peroxidase reaction (DAB, Sigma D-4168).

Modification for BrdU staining

Prior to incubation of sections with primary antibody, DNA was denatured in 2 M HCl for 20 min at 37°C.

Modification for HuC/D staining

Prior to gelatine removal, sections were postfixed with methanol for 20 min at −20°C.

Modification for PCNA staining by peroxidase detection reaction

Antigen retrieval by heating slides to 80–90°C in sodium citrate (10 mM) for 0.5 h was favorable in fish older than 6 months but was omitted in 6-month-olds as it did not influence staining intensity at that age. Endogenous peroxidases were blocked by incubating in 3% H_2O_2 in PBS for 0.5 h. After washing in 0.3% PBX, sections were incubated with the primary antibody.

Modification for BrdU whole mount stainings

Instead of PBX, PDTX (PBS, 1% DMSO, 0.8% Triton-X100) was used.

Microscopy

Light microscopic images were taken with a Zeiss Axioskop microscope equipped with a Sony 3CCD color video camera 12 V. Confocal microscopy was performed with a Zeiss LSM 510 Meta system with a Zeiss Axioplan 2 Imaging MOT (upright) platform. Colocalization studies were performed with sequential scanning to minimize crosstalk between channels. Figures were assembled and edited with Adobe Photoshop and Coral Draw software packages.

Results

Proliferation domains in the zebrafish brain as determined by PCNA and BrdU immunohistochemistry on cross-sections

Because a detailed description of cell proliferation zones in the adult zebrafish brain was missing, we have examined proliferation in zebrafish brains of both sexes in 6-month-old adults (8 fish) by BrdU incorporation experiments. Additionally, we obtained identical results using PCNA immunohistochemistry (Ekström et al., 2001: Wullimann and Puelles, 1999) in 11 adults, 6 months (5 fish), 1 year (2 fish), 1.5 years (2 fish) and 2.5 years (2 fish) of age. With both methods, we have found a highly reproducible pattern of proliferation zones in the brains of all age groups examined. We have determined the destination of newborn cells and their developmental fates and characterized their proliferative behavior. We will begin with a short description of proliferation zones in rostrocaudal order, illustrated by the PCNA material (Fig. 2) and by BrdU immunostained material (Supplementary Fig. 1). An overview of the proliferation zones is given in Fig. 1A.

Olfactory bulb

The paired olfactory bulbs are the rostralmost portion of the telencephalon and protrude ventro-anteriorly from the further caudally located telencephalic areas at an angle of approximately 45°. The interface between the bulbs and the remaining telencephalon is therefore oblique, such that in conventional cross-sections the dorsal telencephalic area overlies the olfactory bulbs in the junctional area.

In cross-sections of the olfactory bulbs, proliferating cells are scattered throughout the bulbs (Figs. 1A, 2A, Supplementary Fig. 1A, zone 1). While cycling cells are dispersed in the anterior part of the bulb, they accumulate along the dorsal surface of the olfactory bulbs near the region of attachment to the dorsal telencephalic area (Fig. 1A arrow, Supplementary Fig. 1A).

Telencephalon

In the dorsal and ventral telencephalic areas (D, V), two distinct proliferation zones can be discerned (zones 2, 3, Fig. 1). Anterior to the anterior commissure, a prominent ventral proliferation zone is located along the ventricular surface of V (zone 2, Figs. 1A, 2B, C, Supplementary Fig. 1B). Close to the olfactory bulb, it spans the ventricular territory adjacent to the ventral and dorsal nuclei of the ventral telencephalic area (Vv, Vd, Fig. 2B). Rostrally, it is especially massive along Vv. Further posteriorly, this proliferation domain leaves the ventricular zone adjacent to Vv and remains restricted to the ventricular zone adjacent to Vd where it becomes less prominent towards the anterior commissure. The dorsal proliferation domain is located in the ventricular zone of D (zone 3, Figs. 1A, 2B–E, O, Supplementary Figs. 1B–E). Due to the everted nature of the teleost telencephalon, parts of the ventricular zone of D come to lie along the dorsal and lateral surface of the brain, covered by an enormously extended roof plate (Fig. 1C). The dorsal proliferation domain is thus located in part along the pial surface of the telencephalon. Proliferating cells are more widely spaced in the dorsal proliferation domain than in the ventral domain. The dorsal proliferation domain extends beyond the level of the anterior commissure. An accumulation of proliferating cells is seen along the lateralmost edge of the dorsal proliferation domain where the roof plate meets the lateral tip of the alar plate (Figs. 1C, 2O). In contrast to the rostral telencephalon where ventral and dorsal proliferation zones are not clearly separated from one another but rather merge at their interface, distinct ventricular proliferation domains are found very closely spaced as separate domains in the diencephalon (zones 4–10, Fig. 1).

Diencephalon

In the preoptic region, a proliferation zone extends in the ventricular zone along the ventral part of the anterior parvocellular preoptic nucleus (PPa; zone 4, Figs. 1A, 2D, Supplementary Fig. 1D) beyond the optic chiasm and tapers off at the level of the postoptic commissure. No proliferation occurs in the dorsal parts of PPa.

Dorsally, ventricular proliferation zones are located along the ventral habenular nucleus (Hav; zone 6), the periventricular pretectal nucleus (PP; zone 7), along the dorsal thalamus (DT; zone 8) and the ventromedial thalamic nucleus (VM; zone 5) of the ventral thalamus (zones 5–8, Figs. 1A, 2E–G, Supplementary Figs. 1E–I). Below the posterior extend of the dorsal thalamic proliferation zone (zone 8), a separate proliferation zone is located along the ventricular surface of the periventricular nucleus of the posterior tuberculum (TPp; zone 9, Figs. 1A, 2F–H, Supplementary Figs. 1H–J). The periventricular hypothalamus is subdivided into dorsal, ventral and caudal zones (Hd, Hv, Hc; Figs. 2F–J). A contiguous proliferation zone spreads into these three subdivisions (zone 10, Figs. 1A, 2F–J, Supplementary Figs. 1I–M). The diencephalic ventricle above

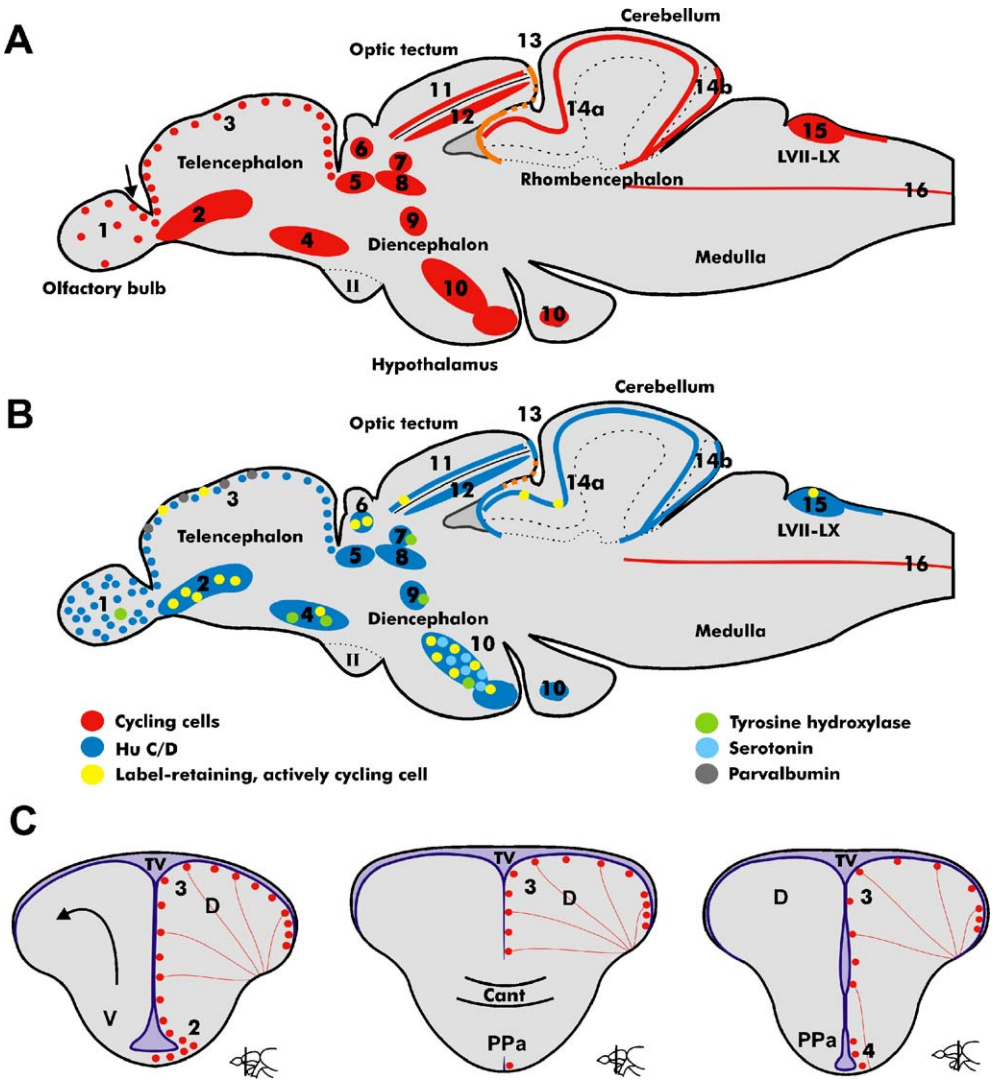


Fig. 1. (A) Schematic drawing of the proliferation zones in the adult zebrafish brain (red areas) numbered by Arabic numerals, lateral view. Olfactory bulb: (1) scattered proliferation in the olfactory bulb. Arrow points to an accumulation of proliferating cells at the junction of the olfactory bulb with the dorsal telencephalon. Telencephalic proliferation zones: (2) ventral and (3) dorsal telencephalic proliferation zones. Diencephalic proliferation zones: (4) preoptic, (5) ventral thalamic, (6) habenular, (7) pretectal, (8) dorsal thalamic, (9) posterior tubercular and (10) hypothalamic proliferation zones. Mesencephalic proliferation zones: (11) tectal and (12) torus longitudinalis proliferation zones. (13) Posterior mesencephalic lamina connects the tectum to the cerebellum. It starts dorsally at the proliferative tectal margin, continues as nonproliferative lamina and becomes proliferative again as it touches the cerebellar surface. Cerebellar proliferation zones: (14a) molecular layer proliferation zone extending through the valvula and copus cerebelli. (14b) Proliferation zone of the cerebellar caudal lobe extending from the ventricular lumen through the granular layer to its surface. Proliferation zones in the medulla oblongata: (15) proliferation zones in the facial (LVII) and vagal (LX) lobes extending caudally into the nucleus of Cajal. (16) Rhombencephalic ventricular proliferation zone extends into the spinal cord. (B) HuC/D-positive neuronal nuclei (blue areas) surrounding all proliferation zones also contain more specialized neuronal subtypes (TH green, 5-HT blue, parvalbumin gray). Newborn cells often differentiate into HuC/D-positive or more rarely into the indicated cell types. Also shown are label-retaining, actively cycling cells residing in proliferation zones of all brain subdivisions (yellow). (C) Schematics of the everted teleost telencephalon showing the ventricular lumen (purple) and the ventricular zone beneath at different anteroposterior levels. Note the accumulation of proliferating cells in the lateral dorsal telencephalic proliferation domain, a likely homologue of the mammalian dentate gyrus proliferation domain.

Hv is sealed off by the posterior tuberal nucleus (PTN; Fig. 2G, Supplementary Figs. 1K, L). Occasionally, proliferating cells are seen within the PTN. The proliferation zone in Hd starts between the PTN and the lateral recess and extends into its tip (Figs. 2G–J, Supplementary Figs. 1K–M). The proliferation zone in Hd merges with the proliferation domain in Hv covering the posterior aspect of ATN (Fig. 2H). Around the posterior recess, nuclei of proliferating cells are not located immediately at the ventricular surface but are found several cell diameters below (Fig. 2I, Supplementary Fig. 1M). In the pituitary, proliferating cells are also seen (Supplementary Figs. 1K, L). The proliferation zone in Hv is continuous with the stalk of the pituitary (Fig. 2G).

Mesencephalon

In the mesencephalon, we recognize two proliferation zones in the optic tectum and the torus longitudinalis (zones 11, 12, Fig. 1). The tectal proliferation zone runs around the margin of the optic tectum (zone 11, Figs. 1A, 2G–J, P, Supplementary Figs. 1H, I, N). It starts anterior–dorsally at the level of the

posterior commissure (Cpost; Fig. 2F), surrounds the tectal commissure further caudally (Ctec; Figs. 2G–I) and continues around the posterior tectal tip. Along the ventral tectal margin, it can be seen only in the posterior third of the tectum (Fig. 1P). Below the tectal commissure, the torus longitudinalis is located in the tectal ventricle. Proliferating cells are found along the ventricular surface of the torus, more or less distinctly separated from the dorsomedian tectal proliferation domain depending on level (zone 12, Figs. 1A, 2G–I, Supplementary Figs. 1H, I). Surrounding the posterior tip of the tectum, a thin sheet of cells seals off the tectal ventricle extending between the caudal tip of the tectum and the cerebellum, the posterior mesencephalic lamina (PML; zone 13, Figs. 1A, 2J, Supplementary Fig. 1O). On the tectal side, it originates from the proliferative tectal margin then continues as a nonproliferative lamina and becomes proliferative again as it meets the valvula cerebelli. At the caudal margin, the PML also extends above the caudal tip of the torus semicircularis. We have not detected proliferation domains in the ventral mesencephalon.

Rhombencephalon

(i) Cerebellum

All three subdivisions of the cerebellum, the valvula cerebelli (Val), the corpus cerebelli (CC) and the vestibulo lateralis lobe (LCa), are built from the two structurally distinct molecular (mol) and granular (gra) layers. Proliferating cells are found in all subdivisions of the cerebellum (zones 14a+b, Fig. 1).

In the valvula and corpus cerebelli, proliferating cells are found scattered throughout the molecular layer while in the granular layer only few PCNA positive cells are seen (zone 14a, Figs. 1A, 2I, J, Supplementary Figs. 1N–P). Cerebellar sections through BrdU incubated zebrafish (2 h, 8 h) show BrdU immunostaining in the molecular layers only while cells in the granular layers of the valvula and the corpus cerebelli do not proliferate. In the midline, where the two halves of the cerebellum have fused across the ventricle during embryogenesis, proliferating cells are oriented along the midline (Figs. 2I, J, Q). They form chains of “radial spokes” in the molecular layers of the valvula up to the dorsal tip of the corpus cerebelli (Fig. 2Q). In the caudal part of the corpus cerebelli, this arrangement of spokes is not detected any more in the molecular layer. Surrounding the rhombencephalic ventricle below the caudal part of the corpus cerebelli, a ventricular proliferation domain emerges (Supplementary Fig. 1P). Proliferating cells can be followed from there across the granular layer of the caudal lobe towards its surface. At the caudal tip of the cerebellum, the caudal lobe forms a cap of granular layer cells covered by proliferating cells (zone 14b, Figs. 1A, 2K, Q, Supplementary Fig. 1Q).

(ii) Medulla oblongata and spinal cord

Caudal to the cerebellum, ventricular proliferation domains are found in the caudal rhombencephalon in the dorsal aspect of the medial facial lobe and in the dorsal tips of the alar plates of the vagal lobe (zone 15, Figs. 1A, 2L, Supplementary Fig. 1R). Further scattered proliferation is apparent in the ventricular zone of the rhombencephalic ventricle. It is this latter ventricular zone proliferation that can be followed caudally into the spinal cord (zone 16, Figs. 1A, 2L, N). Behind the vagal lobe, the ventricular zone proliferation gets a bit stronger at the level of the nucleus of Cajal (NC), where PCNA positive nuclei can be seen close to the ventricular midline of the NC (zone 15, Figs. 1A, 2M, Supplementary Fig. 1S).

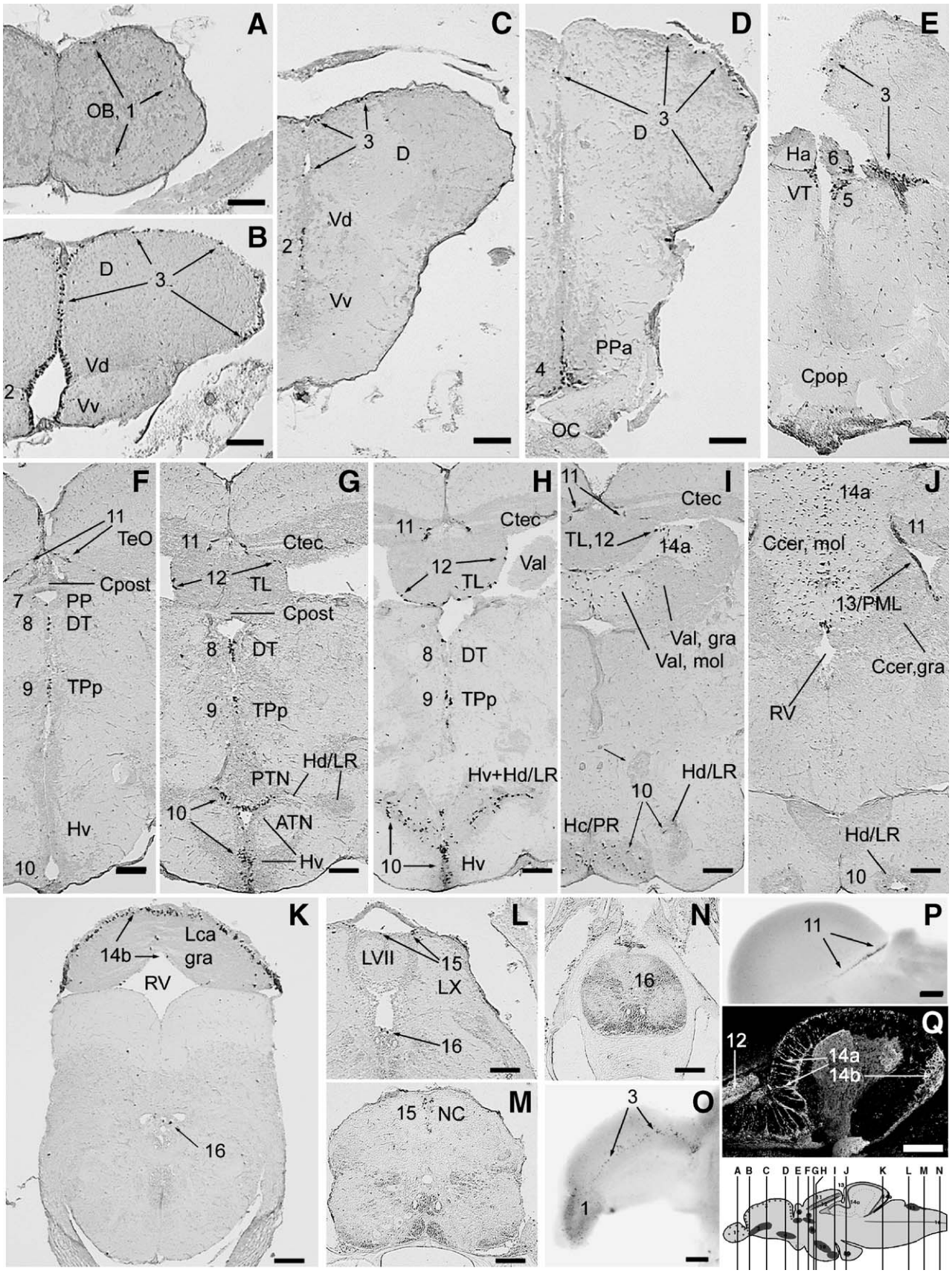
Cells migrate out of the proliferation zones into associated brain nuclei

We examined the mobility of the cycling cells’ progeny. We conducted pulse chase experiments in a total of 9 6-month-old adults in which we followed the distribution of an S-phase marker, either BrdU or IdU, after an initial 48 h pulse and a subsequent chase of 5d (3 fish), 12d (3 fish) or 46d (3 fish). To judge whether the labeled cells had moved away from a proliferation zone, we performed double immunostainings with a second proliferation marker that indicates the position of the proliferation zones at the time of fixation. It is clear from the three timepoints studied that the speed with which newborn cells leave the proliferation zones differs greatly between zones. After the 5d, 12d and 46d chases, we observed an increasing spatial separation of the two markers in the experimental animals as the chase time increased. We observed IdU or BrdU pulse-labeled cells at a distance from the proliferation zones labeled by the second marker (e.g. Fig. 3B, Supplementary Fig. 1N). This finding suggests migration of daughter cells away from the proliferation zones into the mantle layer of the brain. We also found labeled cells that had remained in or in close association to the proliferation zones (e.g. Fig. 3F). The ratio of migrated to stationary pulse-labeled cells differed depending on the proliferation zone under investigation, as did the distances covered by the migratory cells.

Cells moved as little as a few cell diameters in the dorsal telencephalon, but up to several hundred micrometers in the cerebellum according to the nuclear architecture surrounding the proliferation zones. Already in the 5d chase experimental animals, a spatial separation was clearly visible in the ventral telencephalon, the valvula, the corpus and lobus caudalis cerebelli, while in all other proliferation domains, significant overlap of the two proliferation markers was detected (Supplementary Figs. 1A–U). We thus conclude that, in the ventral telencephalon and in the various cerebellar subdivisions, newborn cells move most rapidly away from their sites of origin to their prospective location. While in the ventral telencephalon newborn cells populate the nuclear areas adjacent to the ventricular zone, the movement in the cerebellar subdivisions generally proceeds from the molecular layer to an associated site in the granular layer.

Telencephalon

In the telencephalon of 46d chase experimental animals, most of the progeny of pulse-labeled cells have left the PCNA positive ventricular proliferation zones (zones 2+3) and have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moved into the adjacent or subjacent nuclear areas (Figs. 3A, B). In the dorsal telencephalic area, the new cells have moved a small distance of about 1–2 cell diameters from the ventricular surface into the adjacent and subjacent telencephalic nuclei (Fig. 3A). In the ventral telencephalic area, cells mainly move out radially from the ventricular proliferation zone (zone 2) into the laterally located adjacent nuclei, Vv and Vd (Figs. 3A, B). This predominant direction of movement is already evident after a 5d chase and gets more pronounced in the longer chases (Supplementary Fig. 1B, zone 2). There is some evidence that tangential migration also occurs in the ventral telencephalon. In horizontal sections through the ventral telencephalic proliferation zone of a 5d chase experimental animal, IdU-labeled cells appear to have migrated into the olfactory bulbs from the rostral end of the ventral telencephalic proliferation zone (zone 2, Supplementary Fig. 1U). Furthermore, cells in the ventricular zone of Vv and Vd express PSA-NCAM that has been shown to mark tangentially migrating cells in mice and birds (data not shown, Doetsch and Scharff, 2001; Kaslin et al., in press). However, an exclusively tangential movement of newborn cells along the ventricular walls of the ventral telencephalic proliferation zone may be restricted to its anteriormost tip because of the observed concentric arrangement of pulse-labeled cells around this zone along most of its length (Figs. 3A, B). It is only around the anterior tip of the ventral telencephalic proliferation domain at the interface with the olfactory bulbs where this concentric arrangement of labeled cells around the ventricular zone is less prominent in cross-sections.

Diencephalon

In the ventral diencephalon between anterior and postoptic commissures, most BrdU-labeled cells have migrated laterally into the ventral parvocellular preoptic nucleus surrounding the preoptic proliferation zone (zone 4, Fig. 3D). At the dorsal telencephalic/diencephalic junction, at the level of the anterior part of the ventral thalamic proliferation zone (zone 5), BrdU-labeled cells have migrated into the adjacent ventromedial thalamic nucleus (not shown). Further caudally, below the habenular commissure, BrdU-positive cells have migrated laterally from the PCNA positive habenular proliferation zone (zone 6) into the posterior part of the ventral habenular nucleus (Fig. 3E, Supplementary Fig. 1G). Proliferating cells in the dorsal thalamic and posterior tubercular proliferation zones are not very motile in the 5d chases (zones 8+9, Supplementary Figs. 1H–J). After a 46d chase, PCNA positive and BrdU-positive cells appear as intermingled, non-overlapping populations. Several BrdU-labeled cells do not show migratory behavior but stay close to or in the ventricular zone, closely associated to PCNA positive nuclei, while some have migrated a few cell diameters out into the associated nuclei (Fig. 3F). In the ventral hypothalamus, the BrdU-positive cells have migrated a few cell diameters away from the ventricular proliferation zone (zone 10) into the periventricular ventral hypothalamic nucleus (Figs. 5G, H). In the dorsal hypothalamus around the lateral recess, they have not moved far beyond the PCNA positive population. In the wall of the posterior recess, where the proliferation zone is more spread out, BrdU and PCNA positive cells are again intermingled (not shown, Supplementary Fig. 1M). Labeled cells have also moved into the anterior and posterior tuberal nuclei that are in close contact to the hypothalamic nuclei (Figs. 5G, H).

Mesencephalon

Surrounding the proliferation zone of the optic tectum, a clear migratory behavior of labeled cells can be seen adjacent to the tectal proliferation zone (zone 11) along the posterior third of the optic tectum in the 46d chase experimental animals. Here, a distinct stripe of newborn cells has moved several cell diameters away from the PCNA positive tectal proliferation

Fig. 2. (A–N) Fourteen-micrometer cross-sections of brains of 6 months old adult zebrafish, PCNA immunostained, weak Hematoxylin counterstain. The anteroposterior level of each section is indicated in the lower right hand corner. (O–Q) Brains of 6-month-old adults, BrdU-injected, immunostained as whole mounts after ON survival or (Q) after 24-h survival, halved and fluorescently stained, LSM image, projection. Scale bars, 100 μm. (A) Olfactory bulb with scattered proliferating cells (arrows, zone 1). (B, C) Ventral (2) and dorsal (3) telencephalic proliferation zones at a rostral level (B) and just anterior to the anterior commissure (C). (D) Dorsal telencephalic (3) and preoptic (4) proliferation zones above the optic chiasm. Note the lateral extent of the dorsal telencephalic proliferation zone. (E) Diencephalon at the level of the postoptic commissure with ventral thalamic (5) and habenular (6) proliferation zones overlain by the most posterior part of the dorsal telencephalon with its proliferation zone (3). (F) Diencephalon at the beginning of the posterior commissure with proliferation zones along the periventricular pretectal (7), dorsal thalamic (8), posterior tubercular (9) and ventral periventricular hypothalamic nuclei, overlain by the optic tectum with its proliferation zone (arrows, 11). (G) Medial tectal proliferation zone (11) surrounding the tectal commissure above the torus longitudinalis with its peripheral proliferation zone (12). Along the diencephalic ventricle, the dorsal thalamic (8), posterior tubercular (9) and hypothalamic proliferation zones are seen (10). (H) Posterior to the posterior commissure, the valvula cerebelli protrude into the tectal ventricle. Behind ATN, the ventral and dorsal hypothalamic proliferation zones unite. (I) In the caudal hypothalamus, proliferation continues into the posterior recess (10). In the valvula cerebelli, most proliferation occurs in the molecular layer (14a). (J) This is also true for the corpus cerebelli. The PML seals the midbrain ventricle between tectum opticum and corpus cerebelli. Dorsally, it is continuous with the medial tectal proliferation zone and becomes proliferative again adjacent to the cerebellar granular layer. (K) There is a periventricular proliferation zone below the caudal lobe and also a superficial proliferation zone surrounding the caudal lobe (14b) Zone 14b is tightly associated with the granular layer of the caudal lobe. (K–N) From the medulla, ventricular proliferation continues into the spinal cord (16). In the viscerosensory areas, proliferation is seen ventricularly in the facial and vagal lobes as well as in nucleus of Cajal (M). (O) Accumulation of proliferating nuclei in the lateral part of the dorsal telencephalic proliferation zone (3). (P) Ventral view of the tectal proliferation zone (11). (Q) LSM projection of the medial plane of the cerebellum demonstrating “radial spokes” of cells in that plane. Ccer: corpus cerebelli, Cpost: posterior commissure, Cpop: postoptic commissure, Ctec: tectal commissure, D: dorsal telencephalic area, DT: dorsal thalamus, gra: granular layer, Ha: nucleus habenulae, Hc: periventricular caudal hypothalamus, Hd: periventricular dorsal hypothalamus Hv: periventricular ventral hypothalamus, LCa: lobus caudalis cerebelli, LR: lateral recess, mol: molecular layer, LVII: facial lobe, LX: vagal lobe, NC: nucleus of Cajal, OB: olfactory bulb, OC: optic chiasm, PML: posterior mesencephalic lamina, PP: periventricular pretectal nucleus, PPa: anterior part of the parvocellular preoptic nucleus, PR: posterior recess, PTN: posterior tuberal nucleus, RV: rhombencephalic ventricle, TeO: tectum opticum, TL: torus longitudinalis, TPp: posterior tubercular nucleus, V: ventral telencephalic area, Val: valvula cerebelli, Vd: dorsal nucleus of V, VT: ventral thalamus, Vv: ventral nucleus of V.

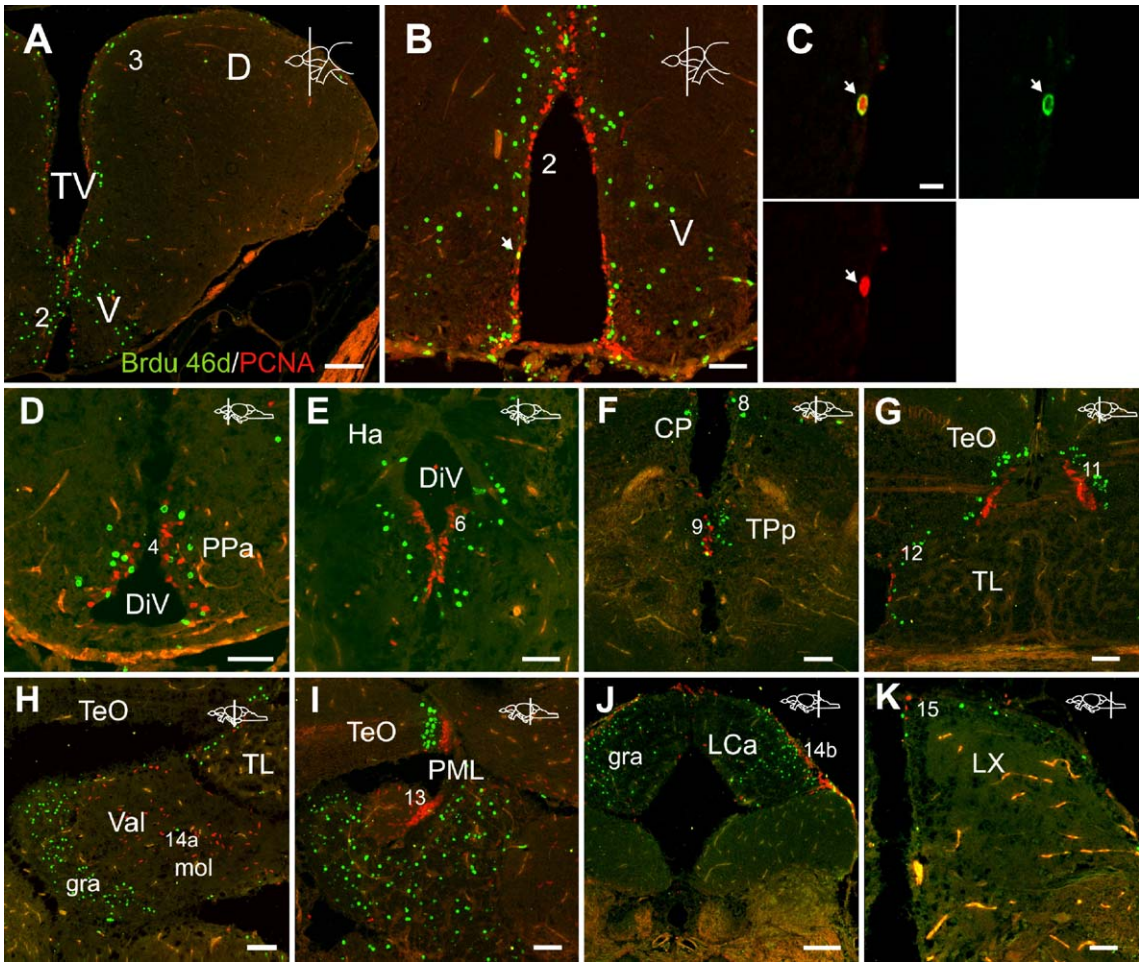

Fig. 3. Fourteen-micrometer cross-sections of a brain of a 7-month-old adult zebrafish double stained for BrdU (green), 46d after an initial 48 h pulse, and PCNA (red) to visualize actively proliferating cells. Anterior–posterior level of sections indicated in the upper right corners. Scale bars, (A) 100 μm, (C) 10 μm, (B, D–K) 50 μm. (A) Telencephalon, overview. (B) Ventral telencephalon. Centrifugal migration of most newborn cells. Label-retaining cells remain in the ventricular zone, a subfraction of those is actively cycling (arrow, BrdU+/PCNA+). (C) Close up of the label retaining, actively cycling cell shown in panel B. (D) Preoptic zone. (E) Ventral habenular proliferation zone. (F) Dorsal thalamic and posterior tubercular proliferation zones. (G) Tectal and torus longitudinalis proliferation zones. (H, I) Valvula cerebelli: differential distribution of migrated cells in the granular and molecular layers. (I) The PML between the TeO and the Val. (J) Peripheral and periventricular proliferation in the lobus caudalis cerebelli. Newborn cells move into the granular layer. (K) In the vagal lobe, newborn cells disperse into the sensory layer. CP: central posterior nucleus of dorsal thalamus, D: dorsal telencephalic area, DiV: diencephalic ventricle, gra: granular layer, Ha: habenulae, LCa: lobus caudalis cerebelli, mol: molecular layer, LX: vagal lobe, PML: posterior mesencephalic lamina, PPa: anterior part of the parvocellular preoptic nucleus, TeO: tectum opticum, TL: torus longitudinalis, TPp: posterior tubercular nucleus, TV: telencephalic ventricle, V: ventral telencephalic area, Val: valvula cerebelli, VT: ventral

zone (Figs. 3G, I). In the 5d chases, the labeled cells in the tectal proliferation zone have not yet appreciably moved from their places of birth (Supplementary Figs. 1H, I, N). We also notice pulse-labeled cells in the torus longitudinalis (Figs. 3G, H) that remain fairly superficial on the torus. Because it is evident that the seemingly continuous dorsal mesencephalic proliferation zone in the dorsal tectum and the torus longitudinalis (Ekström et al., 2001) gives off cells either into the optic tectum or the torus longitudinalis, we interpret this zone as two separate proliferation zones serving two target areas (zones 11+12). As the dorsal tectal proliferation zone becomes continuous with the posterior mesencephalic lamina (PML, zone 13) near the caudal margin of the tectum, it continues to behave like the dorsal tectal proliferation domain, giving off newborn cells towards the tectum opticum (Fig. 3I). The proliferative cerebellar side of the PML does not as clearly give off new cells towards the cerebellar granular layer (Fig. 3I). Further posterior and laterally, the caudal tectal tip and the associated proliferation domains come to lie over the torus semicircularis. At this point, the tectal proliferation domain also gives off newborn cells to the torus semicircularis (Fig. 4N).

Rhombencephalon

A clearcut pattern of migration is associated with the cerebellar proliferation zones (zones 14a+14b). Progeny of proliferating cells are found in the granule cell layer of the valvula and corpus cerebelli as early as 24 h after the initial BrdU pulse. Assuming that cells radially migrate from the molecular layer into the associated granular cell layer, the cerebellum is the brain region where newborn cells migrate most quickly over large distances. Indeed, already 5d after an initial IdU pulse, the labeling pattern observed in molecular and

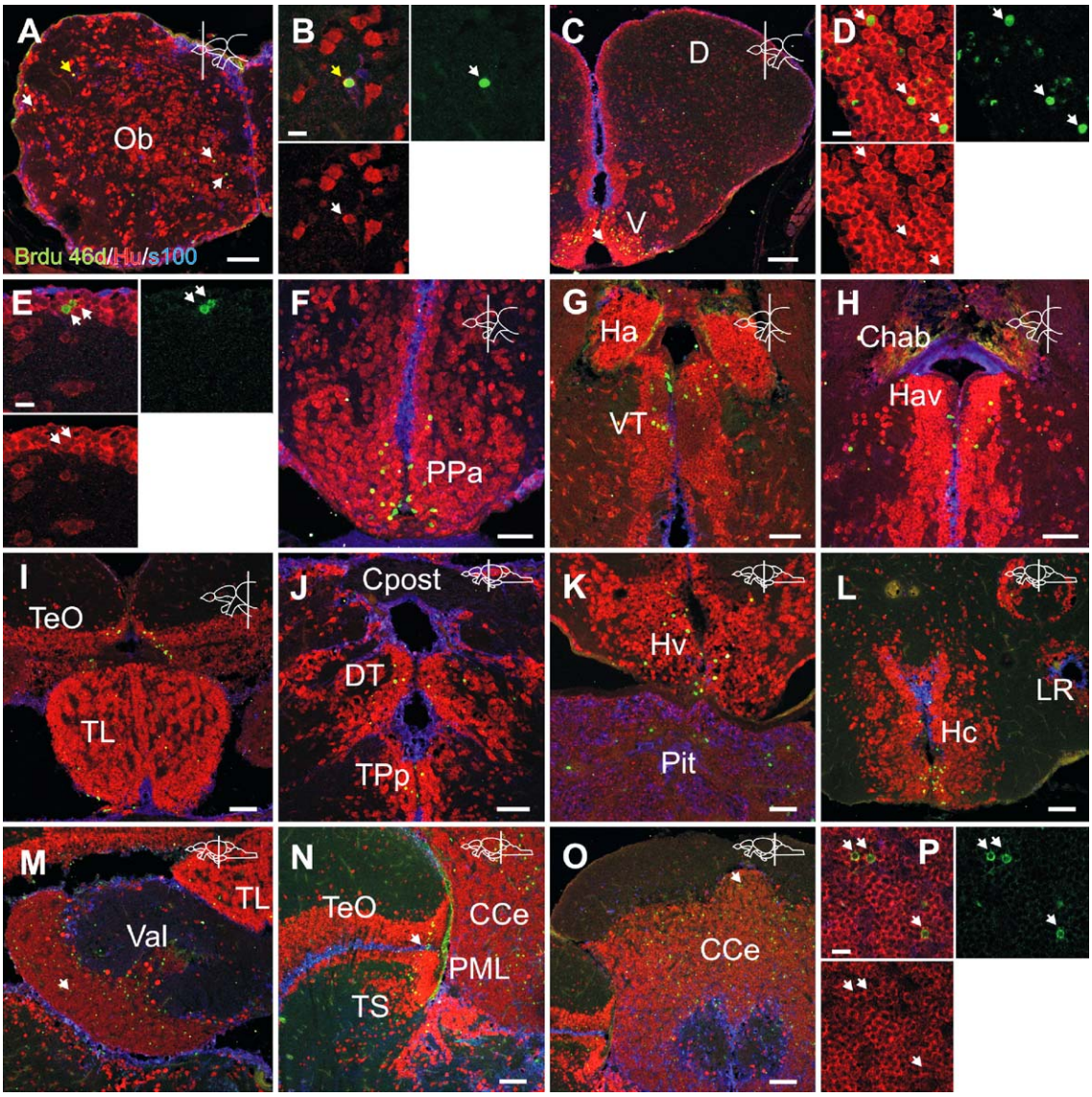

Fig. 4. Fourteen-micrometer cross-sections of a brain of a 7-month-old adult zebrafish stained for BrdU (green) 46d after an initial pulse, HuC/D (red) and S100β (blue). Scale bars: (A, C, F–O) 100 μm, (B, D, E, P) 10 μm. (A, C) Olfactory bulb and telencephalon showing BrdU+ cells in the HuC/D+ nuclear territories (arrows). (B) Close up of a newborn neuron in the olfactory bulb. The yellow arrow in A points to the HuC/D+ cell shown in B. (D, E) Close ups of new HuC/D+ neurons in the ventral and dorsal telencephalon, respectively. BrdU+ cells have also moved into HuC/D-positive nuclei surrounding the ventricular zones in the (F) preoptic area, (G) ventral thalamus, (H) ventral habenular nucleus, (I) optic tectum and torus longitudinalis, (J) dorsal thalamus and posterior tuberculum, (K, L) periventricular hypothalamus and pituitary. (M, N, O) In the valvula and corpus cerebelli, BrdU+ cells (arrows) have moved into the HuC/D+ granular layers where they express HuC/D as seen in the close up of the corpus cerebelli granular layer (P). (N) Adjacent to the posterior mesencephalic lamina, BrdU+ cells have moved into the HuC/D+ nuclear areas of the optic tectum and the torus semicircularis and into the S100β+ ventricular zone (arrow) of the tectum opticum. CCe: corpus cerebelli, Chab: habenular commissure, Cpost: posterior commissure, D: dorsal telencephalic area, DT: dorsal thalamus, gra: granular layer, Ha: habenulae, Hav: ventral habenular nucleus, Hc: periventricular caudal hypothalamus, LR: lateral recess, Ob: olfactory bulb, Pit: pituitary, PML: posterior mesencephalic lamina, PPa: anterior part of the parvocellular preoptic nucleus, TeO: tectum opticum, TL: torus longitudinalis, TPp: posterior tubercular nucleus, TS: torus semicircularis, V: ventral telencephalic area, Val: valvula cerebelli, VT: ventral thalamus.

granular cell layers has completely reversed in the cerebellum (Supplementary Figs. 1N–P). The molecular layer is now almost devoid of labeled cells while they are packed in the granular layer (Fig. 3H). In the lobus caudalis cerebelli, migrated cells are found within the granular layer of the LCa while the proliferative population is seen around its dorsal surface and around the rhombencephalic ventricle (Fig. 3J).

In the vagal lobe, new cells are found in the alar plate sensory nucleus (Morita and Finger, 1985), either somewhat intermingled with the PCNA positive cycling population (zone 15) or further away in a more dorsolateral position or further ventrally along the ventricle (Fig. 2K). New cells are also sometimes found scattered in the vagal lobe fiber layer.

Cells remaining in the proliferation zones

Forty six days after the BrdU pulse, we not only observed migratory cells that had moved out of the proliferation zones,

but also BrdU label-retaining cells within the PCNA positive proliferation zones. We found two populations of BrdU label-retaining cells in the proliferation zones. More frequently, we found cells that were BrdU-positive but PCNA-negative (Figs. 3B, D, F, K), while the nuclei of a few BrdU-positive cells were also labeled by PCNA (Figs. 2B, C). The latter fraction of cells represents label-retaining, actively cycling cells. We have analyzed sections of one brain by confocal microscopy and observed label-retaining, actively cycling cells in the ventral (5 cells) and dorsal (3 cells) telencephalic (zones 2+3; Fig. 1B), the habenular (zone 6, 2 cells, Fig. 1B), the hypothalamic (zone 10, 7 cells, Fig. 1B), the tectal (zone 11, 1 cell, Fig. 1B), the cerebellar (zone 14a, 2 cells, Fig. 1B) and the vagal lobe (zone 15, 1 cell, Fig. 1B) proliferation zones. We have not yet observed label-retaining, actively cycling cells in the preoptic, the ventral and dorsal thalamic and posterior tubercular proliferation domains. The occurrence of label-retaining, actively cycling cells together with PCNA-only-labeled cells suggests that the proliferation zones of the adult zebrafish brain contain different cell types which are characterized by different lengths of their cell cycles.

Different cell types with different cell cycle lengths also exist in the mouse SVZ that contains slowly cycling, label-retaining stem cells and highly proliferative transit amplifying cells (Doetsch, 2003). We propose that the label-retaining, actively cycling cells represent a stem cell pool in the zebrafish brain.

Differentiation pattern

To determine whether newly born cells turn into neurons, we followed the fate of cells that have left the proliferation zones after a 46-day chase. We sectioned 6 individuals from the above described pulse chase experiment and immunostained cryosections with antibodies against BrdU and the general neuronal marker HuC/D (Marusich et al., 1994) as well as the glial marker S100β (Wainwright et al., 2004). HuC/D proteins are expressed from an early neurogenic stage onwards in postmitotic cells that have been committed to a neural fate as well as in mature neurons (Marusich et al., 1994; Barami et al., 1995). In order to visualize whether more specific neuronal phenotypes had been generated besides HuC/D-positive cells during the 46d chase, we utilized antibodies to tyrosine hydroxylase (TH), serotonin (5-HT) and parvalbumin to investigate addition of neurons to the subpopulations defined by these markers. An overview is given in Fig. 1B. Antibodies to TH and 5-HT recognize neurons of the catecholaminergic and serotonergic classes in zebrafish (Kaslin and Panula, 2001). Parvalbumin has been reported to label preferentially interneurons in mammals (Klausberger et al., 2005).

Generally, proliferation zones of all brain subdivisions are surrounded by HuC/D-positive neuronal nuclei into which BrdU-labeled newborn cells have migrated (Figs. 1B, 4). The newborn cells often show HuC/D immunoreactivity suggesting that they are committed to a neuronal fate (e.g. Figs. 4B, D, E, P).

The olfactory bulb is an exception to this general scheme as the HuC/D immunoreactive cell clusters do not surround a proliferation zone but are interspersed with single proliferating cells (zone 1, Fig. 4A). We have detected few BrdU and HuC/D double labeled small granular cells in the olfactory bulb (Fig. 4B). However, their origin is unclear as they could have either originated from mitotic precursors within the bulb or they could have migrated into the bulb from the posteriorly adjacent ventral telencephalic proliferation domain.

All other proliferation zones such as the dorsal and ventral telencephalic zones conform to the general scheme. BrdU/HuC/D double positive cells are detected in the telencephalic neuronal nuclei, up to 20 cell diameters away from the ventral telencephalic proliferation zone (zone 2) in V (Figs. 4C, D) or only 1–2 cells from the dorsal telencephalic proliferation zone (zone 3) in D (Figs. 4C, E). In a similar way, we have also detected BrdU/Hu double stained cell around other proliferation zones: the preoptic (zone 4, Fig. 4F), the ventral habenular (zone 6, Fig. 4H), the dorsal and ventral thalamic (zones 8+5 Figs. 4G, J), the posterior tubercular (zone 9, Fig. 4J), the hypothalamic (zone 10, Figs. 4K, L), the dorsal tectal (zone 11, Fig. 4I), around the torus longitudinalis (zone 12, Fig. 4I), the cerebellar (zone 14a, Figs. 4M–O) and the vagal lobe proliferation zones. A further close up is given in Fig. 4P showing BrdU/HuC/D-positive small granular cells in the granular layer of the corpus cerebelli. In addition, we have noticed S100β-positive glial cells lining the ventricular and the dorsal telencephalic surfaces (e.g. Figs. 4C, E) except in the preoptic, the ventral habenular and ventral hypothalamic areas (Figs. 4F, H, K). S100β-positive cells also surround the molecular layers in the valvula cerebelli and separate molecular from granular layers in the corpus cerebelli (Figs. 4M, O). In the dorsal hypothalamus, the tectum and in the cerebellum, we have also detected single BrdU/S100β double labeled cells (e.g. Fig. 4N). The BrdU/HuC/D double labeled cells by far outnumber the BrdU/S100β double labeled cells, indicating that most of the cells in the proliferation zones are neurogenic and only few are gliogenic.

We have also detected newborn cells expressing distinct neuronal phenotypes. We have immunostained every other section from 5 individuals with BrdU/TH/5-HT. Interestingly, we only detected two BrdU/TH double labeled cells in the olfactory bulbs, despite a significant amount of TH staining in the bulbs (Figs. 1B, 5A, B, Table 1), indicating that addition of newborn TH expressing neurons is rare in the olfactory bulb. Further BrdU/TH double stained cells were found in the preoptic area, posterior tuberculum, pretectum and hypothalamus (Figs. 1B, 5J, K, C, D, E, F, Table 1). We also detected BrdU/serotonin double labeled neurons in the ventral periventricular hypothalamus (Figs. 1B, 5H, I, Table 1). Thus, production of more mature neuronal subtypes is a rare event which nevertheless occurs in the aforementioned brain regions in the adult zebrafish during a 46-day period. Interestingly, not only small granular interneurons are generated but also TH and serotonin expressing cells. These

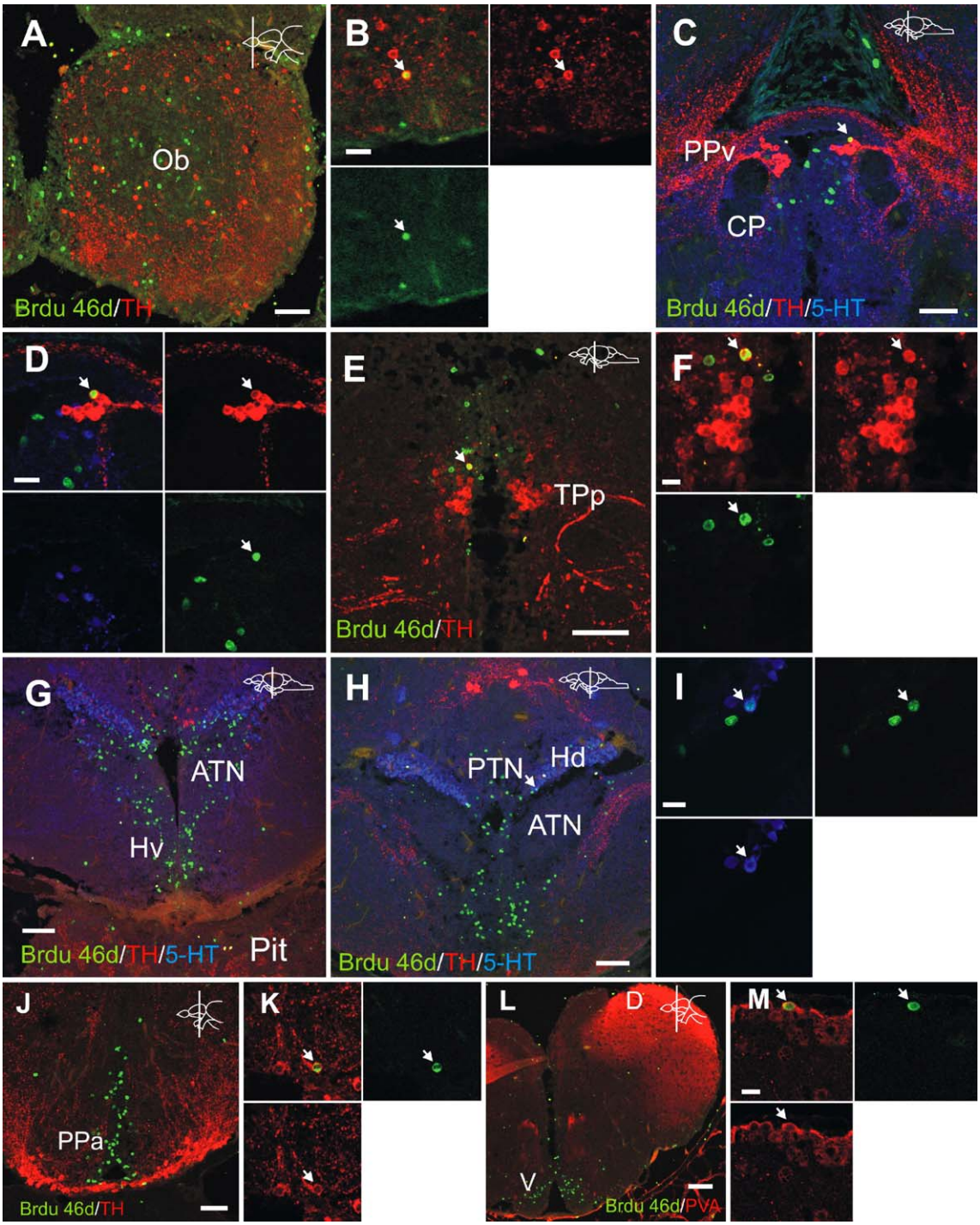

Fig. 5. Fourteen-micrometer cross-sections of brains of 7-month-old adults stained for BrdU, green, 46d after a BrdU pulse and (A–H, J, K) tyrosine hydroxylase (TH, red), (C, G–I) serotonin (5-HT blue), (L, M) parvalbumin (PVA red). Scale bars: A, B, D: 50 μm, C, E, H, I, K, M: 10 μm, F, G, J, L: 100 μm. (A, B) Only little colocalization of BrdU and TH was detected in the olfactory bulb despite abundance of TH+ and BrdU+ cells. (C) Newborn cells differentiated into TH positive neurons in the pretectal nuclear area, (D) close up of (C), (E) in the posterior tuberculum, (F) close up of (E), (J, K) and in the preoptic nucleus. (G–I) Newborn cells move into the 5-HT-positive territory surrounding the periventricular hypothalamus and the ATN (G, H) where they differentiate into 5-HT+ neurons (arrow, I is a close up of H). (L) Newborn cells in the dorsal telencephalic area come to lie in the PVA-positive nucleus. (M) Close up of L. ATN: anterior tuberal nucleus, CP: central posterior nucleus of the dorsal thalamus, D: dorsal telencephalic area, Hd: periventricular dorsal hypothalamic nucleus, Hv: periventricular ventral hypothalamic nucleus, Ob: olfactory bulb, PPa: anterior part of the parvocellular preoptic nucleus, PPv: ventral part of periventricular pretectal nucleus, TPp: posterior tubercular nucleus.

neurotransmitters are characteristic of long projecting neurons in adult zebrafish (Kaslin and Panula, 2001). We have also detected BrdU/parvalbumin double labeled cells in two sectioned individuals in the dorsal telencephalic area (Figs. 1B, 5L, M), whereas we have not detected a BrdU/parvalbumin double positive tectal or purkinje neuron.

Table 1
Newborn aminergic neurons in the adult zebrafish

Region	Olfactory bulbs	Preoptic area	Pretectum	Posterior tuberculum	Hypothalamus	
transmitter	TH	TH	TH	TH	5-HT	TH
Fish 1	0	1	0	1	3	0
Fish 2	0	1	1	0	2	0
Fish 3	1	1	0	1	2	1
Fish 4	1	1	0	1	2	0
Fish 5	0	1	0	1	3	0

Parvalbumin is a characteristic marker of interneurons (Klausberger et al., 2005).

Discussion

Comparison of proliferation domains in adult teleost fish

Proliferation in the brains of adult teleost fish has been known to occur since the pioneering work of Kirsche (1967), but detailed descriptions are available for only few species. We thus limit a comparison of proliferation zones in the adult zebrafish brain to two other teleost species that have recently been described in greater detail. These are the brown ghost *Apteronotus leptorhynchus* (Zupanc and Horschke, 1995) and the stickleback *Gasterosteus aculeatus* (Ekström et al., 2001).

Recently, Zupanc et al. (2005) independently described proliferation zones and neurogenesis in the brain of adult zebrafish. Here, we describe seven additional proliferation zones that were not reported in this study, and for some of the reported zones, we provide a more detailed investigation. Zupanc et al. also suggest in their summary schematics abundant proliferation in the mantle zone of the mesencephalon (optic tectum, torus longitudinalis) which we do not detect and instead find it restricted to ventricular zones only. Moreover, Zupanc et al. report considerable proliferative activity without widespread adult neurogenesis as in the cerebellum and without formation of specific neuronal subtypes as in the diencephalon in long chases. In contrast, we find both widespread neurogenesis and formation of specific neuronal subtypes (parvalbumin, TH and 5-HT-positive).

The proliferation zones we have detected in zebrafish agree surprisingly well with the proliferation zones that have been described in the stickleback (Ekström et al., 2001). We observe proliferation zones in the same locations in zebrafish as in stickleback. Two exceptions are (i) the ventral thalamic proliferation zone which has been reported to be bipartite in the stickleback while we observe just one proliferation zone in the zebrafish and (ii) a deep proliferation zone in the anterior portion of the granular and molecular layers of the valvula cerebelli in the stickleback that is confined to the molecular layer only in the zebrafish. Despite the brackish water dwelling Acanthopterygian stickleback and the tropical freshwater Ostariophysian zebrafish not only inhabit different environments, but have also been placed at opposite ends of the phylogenetic tree of euteleosts (Nelson, 1994), the brain proliferation patterns detected in the two species are surprisingly similar. This is suggestive of a ground plan brain proliferation pattern of euteleosts, perhaps of all vertebrates. We therefore expect to gain general insights into the mechanisms governing adult neurogenesis and growth in the whole group by studying zebrafish proliferation zones.

In contrast, the weakly electric tropical freshwater Ostariophysian brown ghost (Zupanc and Horschke, 1995) shows specific differences in the distribution of adult brain proliferation zones as compared to the zebrafish. The major difference between the two species concerns the location of the dorsal telencephalic and the tectal proliferation zones within the mantle layer of the brain in the brown ghost (Zupanc and Horschke, 1995). We have occasionally observed PCNA or BrdU-labeled cells in the mantle zone of these areas in the zebrafish brain. However, robust, i.e. reproducibly stainable, proliferation zones were neither detected in the mantle zone of the dorsal telencephalon nor in the optic tectum. Instead, proliferation zones were confined to the telencephalic ventricular zone and the tectal margin, respectively. A further important difference is the proliferative behavior of the granular eminence of the cerebellum which is massive in the brown ghost (Zupanc and Horschke, 1995). In contrast, the granular eminences of zebrafish and stickleback are nearly devoid of cycling cells. The specific differences between brown ghost on one side and zebrafish and stickleback on the other side may be attributable to the specific adaptations of the weakly electric brown ghost. Studies on the distribution of adult proliferation zones in species at the base of the teleost radiation will help to clarify this issue.

Patterns of migration and differentiation in the forebrain

In tetrapods, the forebrain subependymal/subventricular layer is one of two neurogenic zones that generates neuroblasts during adulthood that subsequently migrate towards their target areas (reviewed in Doetsch and Scharff, 2001; Kaslin et al., in press). In mammals, tangentially migrating chains of cells, the rostral migratory stream (RMS), are characterized by the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). Cells of the RMS migrate towards the olfactory bulb into which the neuroblasts disperse. There, they differentiate into GABA-ergic and dopaminergic interneurons. The RMS has been found in most mammals that have been investigated to date with the notable exception of humans (Sanai et al., 2004; Quinones-Hinojosa et al., 2006). In a previous pulse chase experiment, it has been shown that the number of BrdU-labeled cells increases within the zebrafish olfactory bulb between 4 days and 4 weeks of chase (Byrd and

Brunjes, 2001). In complete agreement with our data only, very little neurogenesis was reported, as detected by HuC/D and BrdU colocalization after the 4-week chase. Byrd and Brunjes (2001) quantified their results showing that, after a 4-week chase, 4 to 8 times more BrdU-positive cells could be seen than after a 4-day chase. Such an increase in cell number could either be due to a rostral migratory stream into the olfactory bulb as in rodents. Alternatively, the new cells could have been generated in loco during two to three cell cycles of the proliferating cells that we and Byrd and Brunjes (2001) detect throughout the bulb. We have also detected PSA-NCAM in a forebrain ventricular location suggestive of tangential migration along the ventral forebrain nuclei Vd and Vv. However, we note that the majority of the PSA-NCAM arch does not reach the olfactory bulbs but ends just a bit further posteriorly in a very rostral telencephalic position. In birds, it has been described that tangentially migrating neuroblasts in the telencephalic ventricular zone preferentially reach the lobus parolfactorius (Alvarez-Buylla and Kirn, 1997; Alvarez-Buylla et al., 1994) and only a minor fraction reaches the olfactory bulb. Furthermore, we have seen only few newborn cells reach the olfactory bulbs from the ventral telencephalic proliferation zone in our chase experiments while the concentric dispersal and differentiation of labeled cells around the ventral telencephalic proliferation zone argue in favor of additional migratory routes in the zebrafish telencephalon as compared to the rodent situation.

The second neurogenic zone in adult mammals is the dentate gyrus in the hippocampus. In teleosts, the embryologically, neuroanatomically and functionally homologous brain region is the lateral portion of the dorsal telencephalic area (see Kaslin et al., in press for references; Broglio et al., 2005). Already the proliferation zone in the dorsal telencephalon is more pronounced at its lateral edge forming a string of proliferating cells. In the 46-day chase experiments, we have followed the short distance migration of newborn cells in the dorsolateral telencephalon to the subjacent nucleus of D and could detect newborn cells immunopositive for the more general neuronal marker HuC/D and also for the more specific interneuronal marker parvalbumin. We conclude that the dorsolateral telencephalic proliferation zone may be homologous to the hippocampal dentate gyrus proliferation domain in tetrapods.

As we have seen, most BrdU-labeled cells move out of the proliferation zones and differentiate into HuC/D-positive neurons demonstrating that the proliferation zones produce mostly neurons. The majority of these are small and of condensed appearance. Interestingly, newborn TH- and 5-HT-positive cells are also found in several different brain nuclei that contain long projecting aminergic neurons (Kaslin and Panula, 2001). Thus, neurons of different neurochemical phenotype and structure, interneurons and long projecting neurons are continuously generated during adulthood in zebrafish. This is an important difference to the mammalian situation where only few neuronal subtypes are added normally. Furthermore, the addition of catecholaminergic and serotonergic neurons in the adult zebrafish brain is an interesting phenomenon since deficiency of these neurotransmitter systems has been implicated in several human neural disorders.

Migrating, label-retaining and label-retaining, actively cycling cells

Proliferation zones in the adult zebrafish brain persist between 6 months and 2.5 years of age. Thus, cycling cells are present at all times in the proliferation zones during this time interval. From our observation of pulse-labeled cells outside and around all proliferation domains after a 46-day chase, we conclude that labeled daughter cells have left the proliferation zones. To insure the observed maintenance of proliferation zones, these cells have to be replaced.

In the 46-day chase experiments, BrdU pulse-labeled cells were indeed replaced to different extents by PCNA positive/BrdU negative new cells that marked the proliferation zones. Where do the new cells come from? In the adult mouse, two strategies for replacing cycling cells in the proliferation zones of the adult brain have been reported. In the mouse telencephalic subventricular/subependymal zone, asymmetrically dividing stem cells give rise to a population of symmetrically dividing transit amplifying cells. These are highly proliferative and expand the population of cells in the subventricular zone and along the RMS where they assume a neuronal precursor fate and migrate into the olfactory bulb (Doetsch et al., 1997). In the dentate gyrus, a population of asymmetrically dividing stem cells located in the subgranular layer directly gives rise to neuronal precursor cells which migrate into the granule cell layer to differentiate into granule neurons without an intermediate amplifying population (Seri et al., 2001; Hayes and Nowakowski, 2002). In both cases, migration of labeled progenitors away from the proliferation zones dilutes an S-phase label while the resident stem cells self-renew thereby retaining the S-phase label for prolonged periods of time.

Our results indicate that the proliferation zones in the adult zebrafish brain contain at least two distinct populations of cells that differ with respect to their motility and proliferative behavior. In the ventral telencephalic, habenular and cerebellar proliferation zones, the majority of cells belongs to a migratory population that leaves the proliferation zones rapidly within 5 days after the initial IdU pulse. Based on the differentiation behavior and the PSA-NCAM staining of this motile fraction of cells, we propose that this population contains neuronal precursors.

The rapid decrease of the IdU label in the ventral telencephalic, habenular and cerebellar proliferation zones in the 5d pulse chase experiments is accompanied by a strong accumulation of the second S-phase marker, BrdU, which is more pronounced here than in other proliferation zones of the zebrafish brain. This result is suggestive of a rapidly proliferating, perhaps transit amplifying, population of cells in the ventral telencephalic and cerebellar proliferation zones.

The second population of cells consists of a few label-retaining cells that have remained in the proliferation zones. These are of two types: the more abundant BrdU-positive/PCNA-negative cells and the BrdU/PCNA double positive cells. We assume that the former have either withdrawn from the cell cycle and have initiated a differentiation program or represent a quiescent cell-cycle-arrested stem cell population

that would be PCNA-negative (Young, 2004). The less abundant population of cells are label-retaining, actively cycling cells. Label-retaining, actively cycling cells in the zebrafish ventricular zones are candidate stem cells because they fulfil two important criteria of stemness: (i) they remain in the ventricular zone as label-retaining cells for a prolonged time period postadministration as do neural stem cells in the mouse subventricular/subependymal zone (Doetsch et al., 1999). The capacity to retain a S-phase label is also a feature of stem cells found in other tissues (Potten, 2004). A second criterion of stem cells is their capacity to self-renew (Young, 2004; Mikkers and Frisen, 2005). Label-retaining, actively cycling cells have not left their site of origin while proliferating. They thus may fulfil both criteria of stemness. An analysis of marker protein expression in this population of cells is ongoing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.040.

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