# Postembryonic Neurogenesis in Zebrafish (Danio rerio) Brain: Presence of Two Different Systems

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# Postembryonic Neurogenesis in Zebrafish (*Danio rerio*) Brain: Presence of Two Different Systems

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**ABSTRACT**—We analyzed postembryonic neurogenesis in zebrafish brain using the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU). At 1 and 3 weeks after fertilization, BrdU-labeled cells were detected at the brain midline, especially in the telencephalon, optic tectum, hypothalamus, and cerebellum. Cell replication ceased in the telencephalon and hypothalamus by 6 weeks after fertilization, but continued in the optic tectum and cerebellum even in adult fish. Although the area of replication was widely distributed in the tectum and cerebellum of young fish, it seemed to gradually become restricted to the medial and lateral margin of the tectum and the dorsal region of the cerebellum. These areas of replication showed common morphological features, with small, tightly packed spherical cells which were stained very densely by toluidine blue.

In the optic tectum and cerebellum, the areas which were BrdU-positive (or densely stained by toluidine blue) gradually decreased in size relative to the growing brain, but the total volume of these areas in each individual remained unchanged. These results indicate that there are two different neurogenic systems in teleost fish. One system, found in the telencephalon and the hypothalamus, stops replication at an early stage of development; dividing cells in these areas are repressed or removed. The other system, found in the tectum and cerebellum, retains its replicative activity even in adult fish; the number of dividing cells in these areas seems to be maintained during development of the brain.

## INTRODUCTION

It is generally believed that the majority of neural cells, once formed, do not divide again. Cell division occurs only in the initial stages of development, so that the total number of neurons in one individual decreases continually after initial neurogenesis. In higher vertebrates, neurogenesis occurs mainly in the neural tube, and most cell replication stops by the reproduction time (Rakic, 1985). Postnatal neurogenesis has been observed only in limited brain regions, like the hippocampus, and the number of replicating cells is very low (Bayer *et al.*, 1982; Corotto *et al.*, 1993; Kaplan and Bell, 1983; Lois and Alvarez-Buylla, 1993). The various domains of the central nervous system are built by cell proliferation, migration, and apoptosis of post-mitotic neurons (Raff, 1996). In contrast, in lower vertebrates such as the zebrafish (*Danio rerio*), not only body weight but also the weight of the brain continues to increase until death. It has therefore been proposed that, in such organisms, further neural cell replication must occur somewhere in the brain at late stages of brain development.

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In the present study, we observed the development of zebrafish brain. In addition to morphological analysis of the developing brain, we carried out an extensive study of neural cell division using BrdU incorporation. Our results indicate that neurogenesis in the zebrafish telencephalon is similar to higher vertebrate neurogenesis, in that cell replication ceases at an early stage. However, neurogenesis in the tectum and cerebellum is very different; the dividing cells in these areas seem to retain the ability to replicate throughout the animal's lifetime.

## MATERIALS AND METHODS

### Animals

Zebrafish were kept and raised according to standard conditions (Westerfield, 1995; Tomizawa, 2000). Fish were maintained in a laboratory breeding colony at 28.5°C on a 14 hr light / 10 hr dark cycle. Embryos and larvae were obtained by natural mating under standard conditions (Westerfield, 1995). Embryos were collected from fish allowed to breed according to a natural spawning cycle, and were raised at 28.5°C in fresh tank water. Embryos and larvae were staged according to time after fertilization (Kimmel, 1995). Total length was determined as an animal's longest linear dimension.

### BrdU Labeling

Larvae and adults were bathed for 24 hr in a solution of 10 mM BrdU (Tokyo Kasei) in fish water at 28.5°C. Thereafter, larvae and

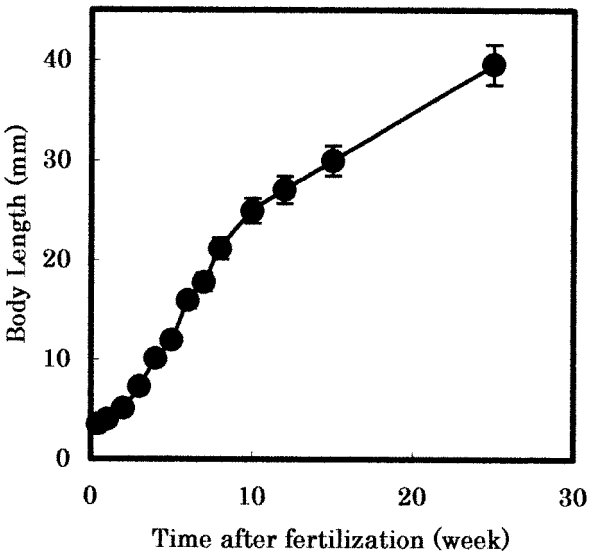
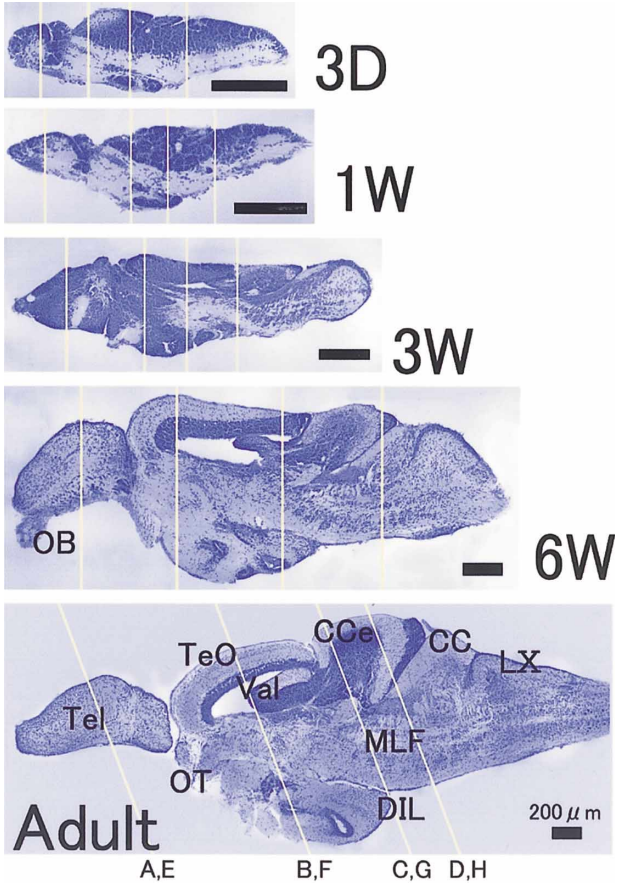



**Fig. 1. Growth of Zebrafish** Plot of zebrafish growth showing total length (mm) versus age (weeks). Zebrafish were staged according to days and weeks after fertilization. Total length was determined as the longest linear dimension of each fish. Fish length was ~3.5mm at hatching, increasing to ~40mm in adult fish (about 25 weeks after fertilization).




adults were either fixed immediately or returned to fresh water for various periods of time in order to follow the fate of labeled cells. Samples were prepared for BrdU detection according to the following protocols.

**Tissue Preparation for Immunohistochemistry**

Tissue preparation was performed by the method of Westerfield (1995). Zebrafish (larvae and adults) were anaesthetized on ice and

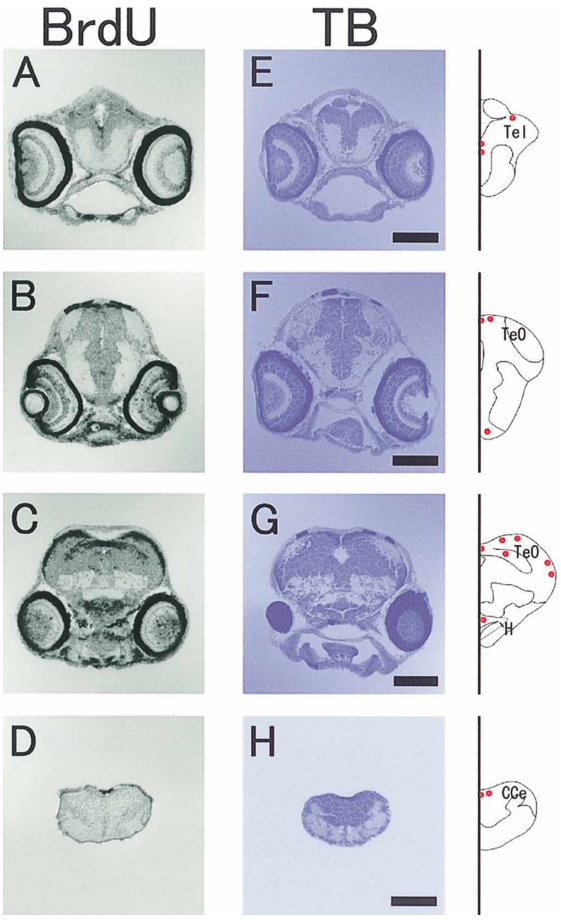



**Fig. 3. Transverse Sections 3 Days after Fertilization (hatching stage)** The left-hand panels (A-D) show transverse sections stained with anti- 5-bromo-2'-deoxyuridine (BrdU) antibody. The middle panels (E-H) show similar transverse sections stained with toluidine blue. The levels at which the sections were taken is shown in Fig. 2. The right-hand panel shows a schematic representation of the distribution of BrdU labeled cells. The locations of labeled cells are indicated by dots. Tel, telencephalon; TeO; optic tectum; H, hypothalamus; Cce, cerebellar corpus. Scale bars,100 μm.

**Fig. 2. Development of Fish Brain in Several Stages** Sagittal sections of whole fish brain with rostral side to the left. Sections of larvae (3 days, 1 week, 3 weeks, 6 weeks) and adults were stained with toluidine blue. The vertical line indicates sectioning levels corresponding to the sections shown in Figs. 3–7 (A–H). 3D, 3 days post fertilization (hatching stage);1W, 1 week after fertilization; 3W, 3 weeks after fertilization; 6W, 6 weeks after fertilization. OB, olfactory bulb; Tel, telencephalon; TeO, optic tectum; OT: optic tract; Val, lateral division of valvula cerebelli; CCe, cerebellar corpus; CC, cerebellar crest; L, vagal lobe; MLF, medial longitudinal fascicle; DIL, diffuse nucleus of the inferior lobe. Scale bars, 200 μm.

brain attached to skull bone was fixed in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% freshly depolymerized paraformaldehyde and 4% sucrose at 4°C overnight (Nakayasu *et al.*, 1989, 1991). Isolated brains (adults) or heads (larvae) were washed gently in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% sucrose. The tissues were embedded in a 1.5% agar solution containing 5% sucrose, immersed in a 30% sucrose solution overnight for cryoprotection and stored at 4°C until sectioning. The tissues were cut into 20 μm sections on a rotary microtome and collected on APS (3-Aminopropyltriethoxysilane) coated slides and dried at 60°C for 10 min.

**Immunohistochemical Staining**

Sections were treated with 0.3% $H_2O_2$ in methanol for 15 min in order to inhibit endogenous peroxidase activity, washed thoroughly with PBS (pH 7.3) and rinsed with distilled water. The sections were subjected to antigen retrieval using microwave radiation (see below) in order to amplify weak BrdU signals (Van de Kant *et al.*, 1990; Dover and Patel, 1994). Following antigen retrieval, sections were rinsed in distilled water followed by PBS (pH 7.3), then soaked in PBS/Tween 20/DMSO solution (PBS containing 0.2% Tween 20 and 1% DMSO) containing 0.1% TritonX-100 for 5 min and washed thoroughly with the same buffer without TritonX-100. Sections were then treated with 2 M HCl for 60 min at room temperature and rinsed several times in PBS/Tween 20/DMSO. Nonspecific protein binding sites were blocked by incubation for 30 min in PBS/Tween 20/DMSO containing 2% Tween 20. The sections were incubated with a 1:100 dilution of mouse monoclonal anti-BrdU antibody (Sigma) in PBS/Tween 20/DMSO at 4°C overnight. After thorough washing with the same buffer, the sections were incubated with peroxidase-conjugated goat anti-mouse IgG antibody (1:100, Jackson Immuno Research) in PBS/Tween 20/DMSO for 2 h at room temperature, washed four times for 10 min each with the same buffer and once for 5 min with 0.1 M $PO_4$ (pH 7.3) and then soaked in diaminobenzidine (DAB) heavy metal staining solution (0.04% DAB, 0.1 M Tris-HCl (pH 7.4), 0.5% DMSO and 0.45% $Ni(NH_4)_2(SO_4)_2$) for 10 min. The peroxidase reaction was initiated by the addition of $H_2O_2$ at a final concentration of 0.003%. The enzymatic reaction was stopped by washing with 0.1 M $PO_4$ (pH 7.3) and then with distilled water, and the sections were dehydrated through an ethanol series (50, 75, 95,100,100%), cleared in xylene and mounted with Permount.

**Antigen Retrieval**

Antigen retrieval was performed according to established methods (McNicol and Richmond, 1998; Ferrier *et al.*, 1998; Loup *et al.*, 1998; Werner *et al.*, 1996; Shi *et al.*, 1991, 1997). The sections were washed in distilled water after blocking endogenous peroxidase activity and then transferred to 100 ml of retrieval solution (10 mM sodium citrate buffer, pH 6.0) in a microwave-safe container. The container was placed in a microwave oven with a rotating plate and the solution was heated to boiling at a power setting of 500 W (90–120 sec). Irradiation was stopped when the fluid boiled, and the sections

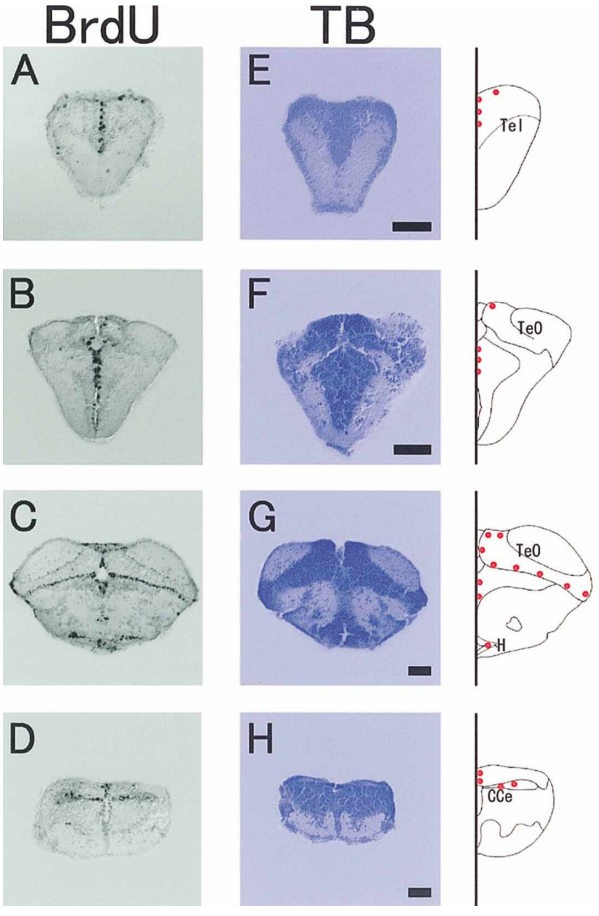



**Fig. 4. Transverse Sections 1 Week after Fertilization** See legend to Fig. 3.

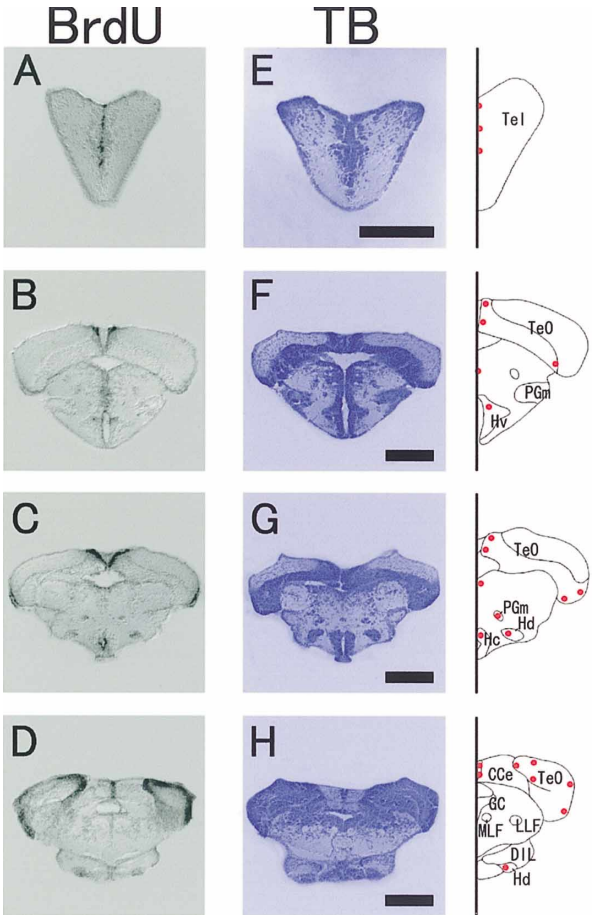



**Fig. 5. Transverse Sections 3 Weeks after Fertilization** See also legend to Fig. 3. Tel, telencephalon; TeO, optic tectum; PGm, medial preglomerular nucleus; Hv, ventral zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hc, caudal zone of periventricular hypothalamus; Cce,cerebellar corpus; GC, central gray; MLF, medial longitudinal fascicle; LLF, lateral longitudinal fascicle; DIL, diffuse nucleus of the inferior lobe. Scale bars, 200 μm.

were left for 5 min in the hot solution. The solution was then heated to boiling again, and the slides were allowed to cool in the retrieval solution to room temperature before further processing for immunohistochemistry.

### Toluidine Blue Staining

Toluidine blue staining was used for analysis of brain tissue morphology. Sections were soaked for 3 min in 0.05% toluidine blue O (Sigma) in a solution consisting of 3.5 ml of 0.1 M citric acid plus 16.5 ml of 0.2 M $NaH_2PO_4$ (pH 7.0), then washed with distilled water, dehydrated through an ethanol series, cleared in xylene and mounted with Permount as above.

### Viewing and Processing Images

Thin sections were viewed with a Zeiss Axiophot microscope using an appropriate filter set. Photographs were taken with a CCD camera (Photometrics KAF-1400) using V for Windows (Photometrics), processed in Photoshop (Adobe) and printed using a Pictrography 3000 (Fuji Film). The dimensions of the whole brain and of replicating areas were measured using Photoshop and Microsoft Excel. Total brain volumes were calculated from these measurements and the thickness of the sections.

## RESULTS

### Development of Zebrafish Brain

Zebrafish embryos hatched approximately 3 days after fertilization. At this stage, the total length of an average fish was about 3.5 mm. The fish increased gradually in length until they reached adulthood, and an average length of 40 mm, about 25 weeks after fertilization (Fig. 1). Brain length from rostral side to caudal side was about 750 μm at 3 days and about 4 mm in the adult. Although the optic tectum and cerebellum were not clearly defined up to 1 week after fertilization, these structures were clearly distinguishable at 3 weeks (Fig. 2). The outer layer of the tectum was not visible at this stage, but was clearly detectable at 6 weeks. Development of the cerebellum was poor at 3 weeks. Valvula cerebelli extended beneath the tectum at 6 weeks and gradually increased its volume. It should be noted that the area of granular cell layer in the cerebellum increased during the later stages of development of the brain (Fig. 2).

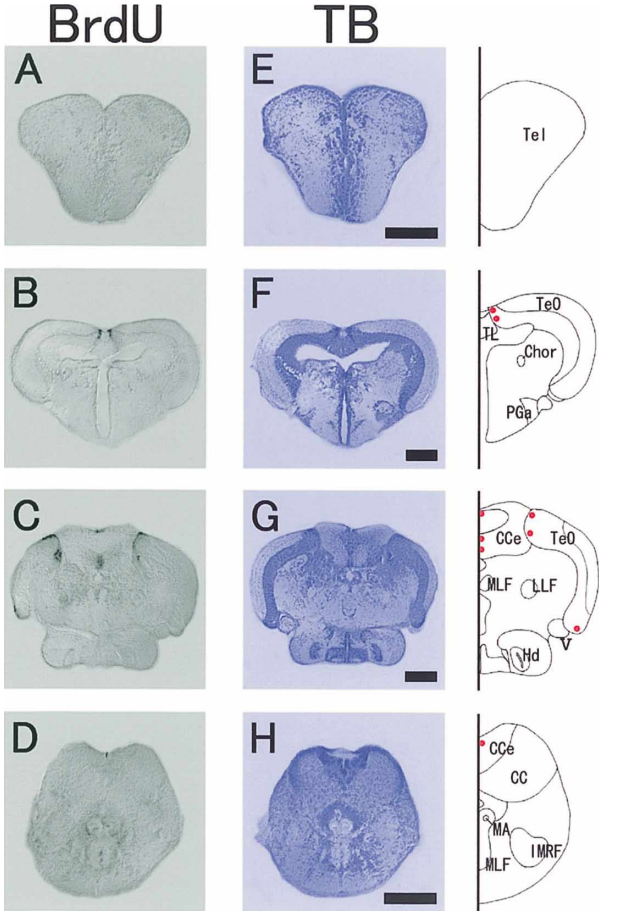



**Fig. 6. Transverse Sections in 6 Weeks after Fertilization** See also legend to Fig. 3. Tel, telencephalon; TeO, optic tectum; TL, longitudinal torus; Chor, horizontal commissure; Pga, anterior preglomerular nucleus; CCe, cerebellar corpus; MLF, medial longitudinal fascicle; LLF, lateral longitudinal fascicle; Hd, dorsal zone of periventricular hypothalamus; áX, trigeminal nerve; CC, cerebellar crest; MA, Mauthner axon; IMRF, intermediate reticular formation. Scale bars, 200 μm.

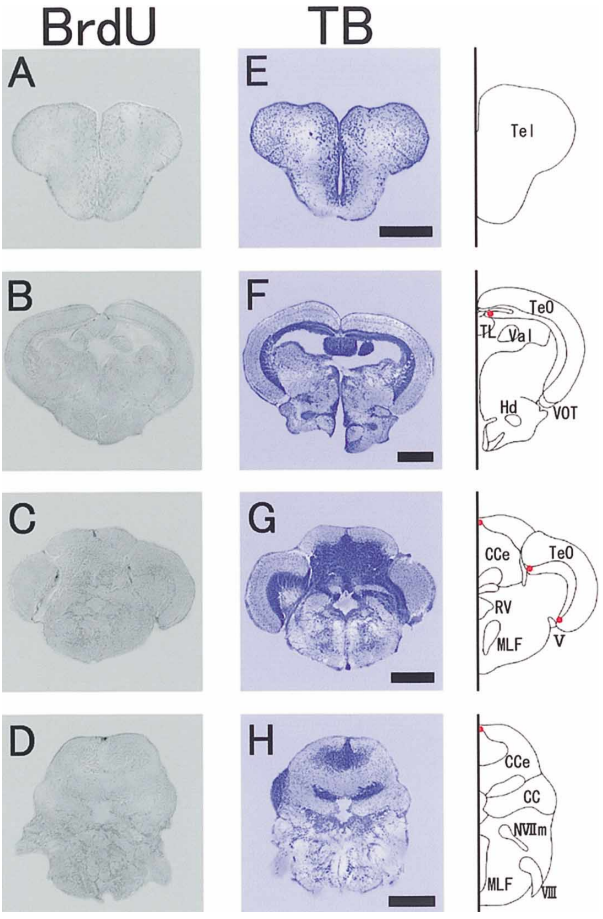



**Fig. 7. Transverse Sections in Adult** See also legend to Fig. 3. Tel, telencephalon; TeO, optic tectum; TL, longitudinal torus; Val, lateral division of valvula cerebelli; Hd, dorsal zone of periventricular hypothalamus; VOT, ventrolateral optic tract; Cce, cerebellar corpus; RV, rhombencephalic ventricle; MLF, medial longitudinal fascicle; V, trigeminal nerve; CC, cerebellar crest; NVIIm, facial motor nucleus; VIII, octaval. Scale bars, 400 μm.

The facial and vagal lobes were very difficult to distinguish at 3 weeks, but became obvious at 6 weeks (about 200–300 μm in size) and grew continuously, reaching 600–700 μm in the adult brain. The number of neurons in these areas also seemed to increase over the same period of time.

In contrast, neurons became apparent in the brainstem as early as 3 weeks and gradually increased in number until 6 weeks post-fertilization. The brainstem of young fish (6 weeks) was almost identical to that of adult fish.

**BrdU Incorporation in Developing Zebrafish Brain.**

**3 days after fertilization** Figure 3 shows transverse sections 3 days after fertilization (hatching stage). BrdU labeling was observed in the midline of the telencephalon (Tel) (Fig. 3A). In the optic tectum (TeO), BrdU labeling was observed in the superficial, dorsal, and lateral zones of the tectal plate (Fig. 3B, C). Labeling was also observed in an area considered to be the hypothalamus. In the cerebellar corpus (CCe), labeling was concentrated in the dorsomedial region (Fig. 3D).

**1 week after fertilization** Figure 4 shows transverse sections 1 week after fertilization. In the Tel, labeled cells were located in the midline (Fig. 4A). At the level of the midbrain, BrdU labeling was observed also in the midline, in the hypothalamus, and along the ventral margin of the TeO (Fig. 4B, C). In the tectum, the boundary of the periventricular gray zone (PGZ) and upper layers was clearly defined. In the CCe, BrdU labeling was observed in the dorsomedial region (Fig. 4D).

**3 weeks after fertilization** Figure 5 shows transverse sections 3 weeks after fertilization. In the Tel, BrdU labeling was observed along the midline (Fig. 5A). In the midbrain, BrdU labeling was observed in several areas, including the medial preglomerular nucleus (PGm) and the caudal (Hc) and dorsal (Hd) zones of the periventricular hypothalamus, in addition to the midline. By this time the hypothalamus had begun to separate into distinct caudal, dorsal and ventral zones. In the TeO, labeled cells were found only in the lateral, caudal, and medial margins of the tectum (Fig. 5B, C, D). In the CCe, BrdU labeling was observed in the midline of the granule cell layer (GCL) (Fig. 5D). The GCL and molecular cell layer (MCL)

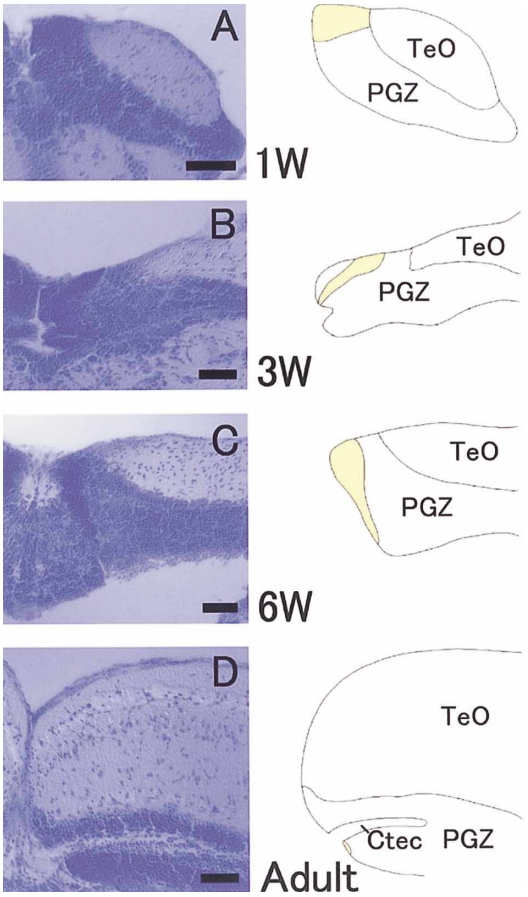



**Fig. 8. The Developing Marginal Zone of the Optic Tectum** Sections are shown as photomicrographs stained with toluidine blue and as schematic drawings. In each schematic drawing, the colored area represents the marginal zone of the optic tectum (TeO). This area belongs to the periventricular gray zone (PGZ) and is occupied by tightly packed cells. A, 1 week after fertilization; B, 3 weeks after fertilization; C, 6 weeks after fertilization; D, Adult. Ctec, tectal commissure. Scale bars, 50 μm.

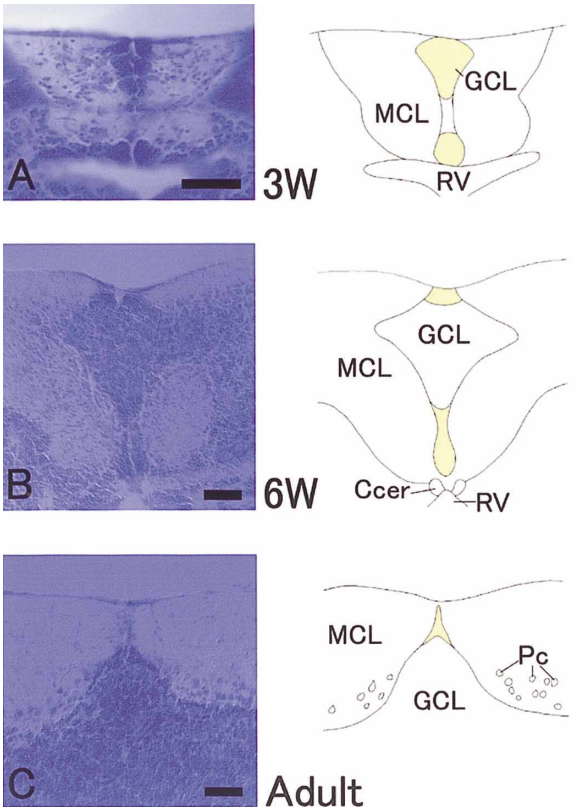



**Fig. 9. The Developing Dorsal Region of the Cerebellar Corpus** Sections are shown as photomicrographs stained with toluidine blue and as schematic drawings. In each schematic drawing, the colored area represents the dorsal and ventral zones of the cerebellar corpus (CCe). This area is formed by the granule cell layer and is occupied by tightly packed cells. A, 3 weeks after fertilization; B, 6 weeks after fertilization; C, Adult. MCL, molecular cell layer; GCL, granule cell layer; RV, rhombencephalic ventricle; Ccer, cerebellar commissure; Pc, Purkinje cell. Scale bars, 50 μm.

of the cerebellum could be clearly distinguished; labeling was restricted to the GCL, and not observed in the MCL.

**6 weeks after fertilization** Figure 6 shows transverse sections 6 weeks after fertilization. BrdU labeling was no longer observed in the Tel (Fig.6A) or in the hypothalamus. Furthermore, in the TeO, the labeled zone had become restricted to the lateral and medial margins of the tectum (Fig. 6B, C). In the CCe, BrdU labeling was concentrated in the dorsomedial and ventromedial zone of the GCL, which protruded at the midline into the MCL (Fig. 6C, D).

**Adult** Figure 7 shows transverse sections of adult fish brain (about 25 weeks after fertilization). BrdU labeling was observed only in restricted regions of the optic tectum and cerebellum. The active region was restricted to the lateral and medial margins of the tectum (Fig. 7B, C) and the dorsomedial zone of the GCL (Fig 7C, D).

The distribution of active cellular replication at various stages is summarized in Figure 8.

**Morphology of the Replicating Zone**

**Optic tectum** We have paid particular attention to the histological structure of the tectal marginal zone. This area belongs to the PGZ, but the marginal zone of the PGZ differed structurally from the remainder of the PGZ. A prominent morphological feature of the tectal marginal zone was very tightly packed cells which stained heavily with toluidine blue (Fig. 9). Both BrdU-positive and heavily toluidine blue-stained areas were distributed widely in the tectum at early stages of development, but were gradually restricted to a small region of the PGZ as development progressed.

**Cerebellar corpus** The cerebellum is composed of 2 major layers, the GCL and the MCL, which were clearly distinguishable after 3 weeks (Fig. 10). The actively replicating area in the cerebellum was also occupied by tightly packed cells which were strongly stained by toluidine blue. In the adult, this area was restricted to the dorsal region of the GCL. The area of the active zone in a single section gradually decreased during development.

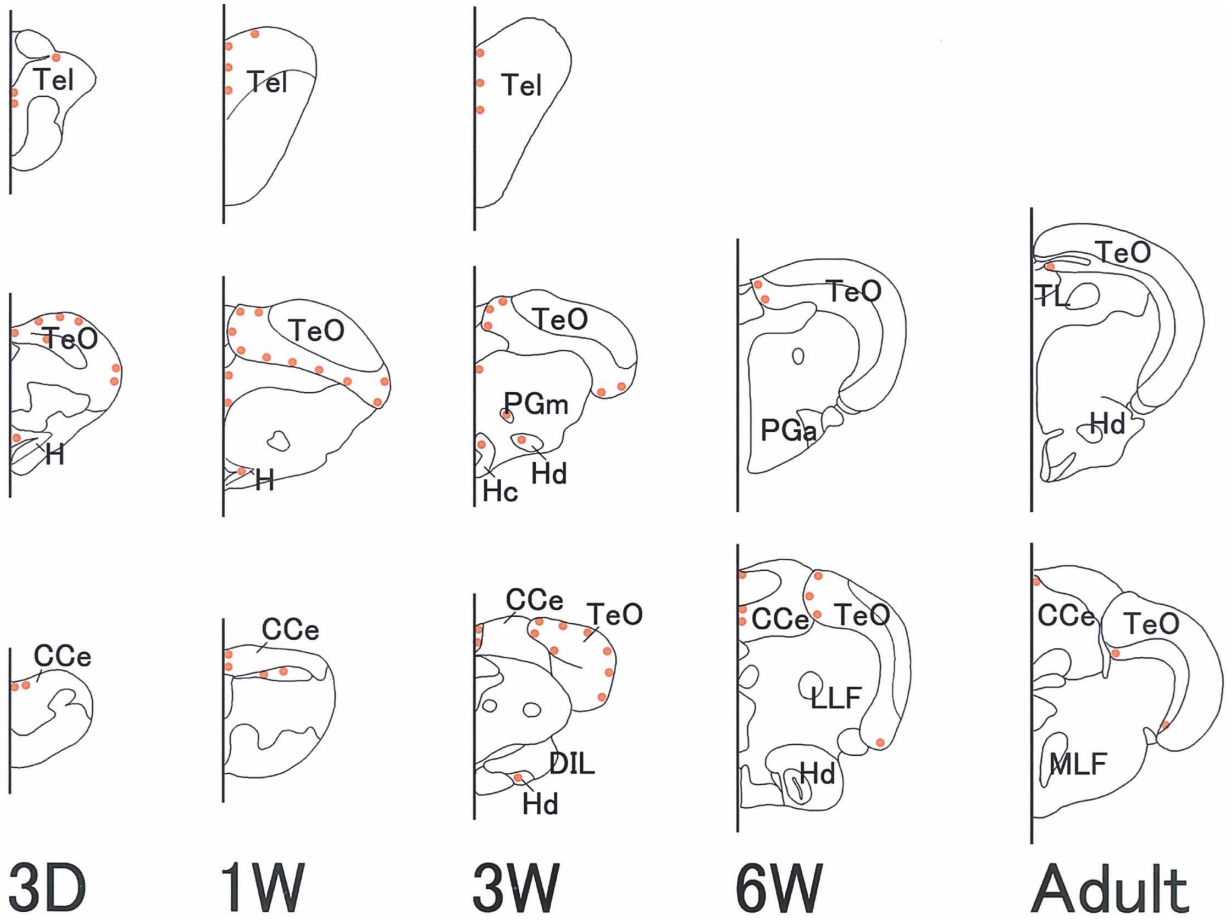



**Fig. 10. Schematic Representation of the Distribution of BrdU Labeled Cells at Various Stages** The locations of BrdU labeled cells are indicated by dots. In the telencephalon, cell proliferation had ceased by 3 weeks post-fertilisation. In contrast, cell replication in restricted areas of the optic tectum and cerebellar corpus continued to adulthood. 3D, 3 days after fertilization (hatching stage); 1W, 1 week after fertilization; 3W, 3 weeks after fertilization; 6W, 6 weeks after fertilization. Tel, telencephalon; TeO, optic tectum; TL, longitudinal torus; H, hypothalamus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; PGa, anterior preglomerular nucleus; PGm, medial preglomerular nucleus; Cce, cerebellar corpus; DIL, diffuse nucleus of the inferior lobe; MLF, medial longitudinal fascicle; LLF, lateral longitudinal fascicle.

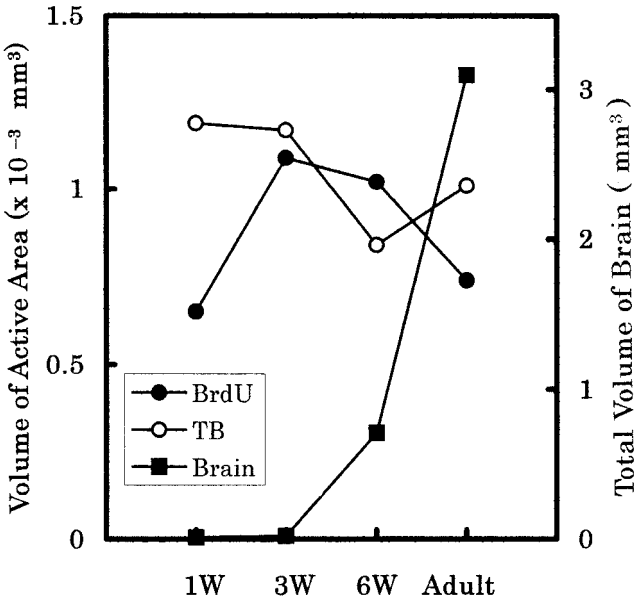



**Fig. 11. Change of Total Volume of Replicating Zone in Cerebellum during Brain Development** Total brain volume (squares) and the total volumes of the BrdU-positive region (closed circles) and the region densely stained by toluidine blue (open circles) in the cerebellum were calculated from serial 20 μm cross-sections.

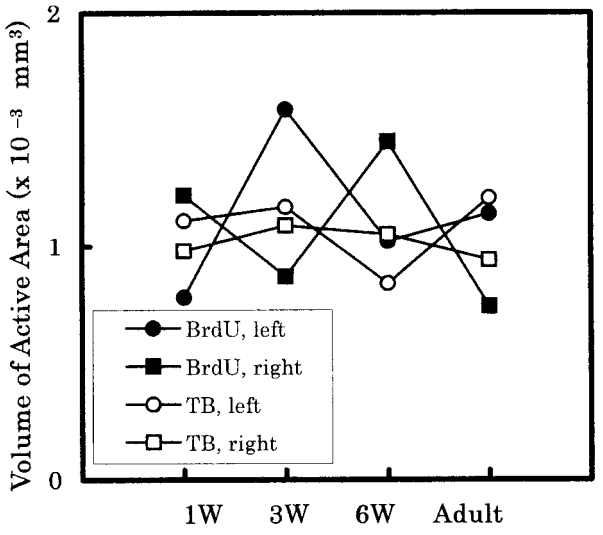



**Fig. 12. Change of Total Volume of Replicating Zone in Left and Right Tectum during Brain Development** The total volumes of the BrdU-positive region (closed circles or squares) and the region densely stained by toluidine blue (open circles or squares) in the tectum were calculated from serial 20 μm cross-sections. Values obtained in the left tectum are indicated by circles and values from the right tectum are indicated by squares.

### Change in Volume of Replicating Zone

Although the relative area of the active zone gradually decreased, the body length of the fish continuously increased, with the overall result that the total volume (or total number of dividing cells) in the replicating area did not change during development. Figure 11 shows that the total volume of the brain increased dramatically, and that the calculated volume of the BrdU-positive (or toluidine-blue dense) area in the cerebellum did not change during development. The same observation, that the total volume of the active zone did not change during development, was made on both the left and right sides of the tectum (Fig. 12).

## DISCUSSION

### Immunohistochemistry using BrdU

It has long been widely accepted that vertebrate neurogenesis occurs only during certain limited stages of brain development and takes place mainly in the neural tube, whence newly synthesized cells migrate to their final positions (Bayer *et al*., 1982; Kaplan and Bell, 1983; Rakic, 1985; Corotto *et al*., 1993;). This idea was partially challenged by the observation that neurogenesis continues even in adults (Lois and Alvarez-Buylla, 1993; Nguyen, 1999). However, the site of neural cell replication was usually limited and the rate of replication slow. In the case of lower animals such as fish, body length and weight increase continually during the animal's lifetime, as does total brain mass. Therefore, adult neurogenesis might be expected to be more rapid and present in a greater area of the brain in fish than in higher vertebrates.

In the present study, we visualized newly synthesized zebrafish neural cells by BrdU labeling and immunohistochemistry, and compared these results with detailed morphological analyses of brain tissues at various stages in the development of the fish.

### Nature of the Replicating Zone

We found that neurogenesis continues in the tectum and cerebellum, even in adults. In contrast, replication in the telencephalon and hypothalamus ceased at a much earlier stage. However, the replicating areas in the adult brain were limited to the margins of the tectum and the dorsal region of the cerebellum; these areas were also active at 3 and 6 weeks post-fertilization. These observations are consistent with previous research (Zupanc and Zupanc, 1992; Zupanc *et al*., 1996; Van Nguyen *et al*., 1999).

Replication of neurons in the marginal zone of the tectum was reported previously by Raymond and Easter (1983). Although this marginal zone belongs to the PGZ, it is histologically distinct from the surrounding PGZ structures. The replicating zone is densely stained by toluidine blue and the cells are tightly packed.

In the cerebellum, the dorsomedial and ventromedial zones of the GCL showed mitotic activity. These zones form the tip of the GCL, which protrudes at the midline into the MCL. This protruding "tip" structure is described by Zupanc *et al*. (1996) as being composed of tightly packed cells which are densely stained by toluidine blue. This description is entirely consistent with our observations.

### Two Different Neurogenic Processes

Figures 11 and 12 show that the volumes of both the BrdU-positive and the densely toluidine blue-stained areas did not change during brain development. Although not all

BrdU-positive cells are actively dividing, the number of BrdU-positive (or toluidine blue dense) cells is expected to be directly proportional to the number of dividing cells. Assuming this to be true, our results suggest that the number of dividing cells in the tectum and cerebellum did not change during development, although the distribution of the dividing cells did change. The active zone covered a wider area in early stages (1 and 3 weeks) and was gradually restricted as development proceeded. Our results also suggest that the dividing cells in the tectum and cerebellum maintained replicative activity throughout development. In contrast, cell replication in the telencephalon and hypothalamus stopped by 6 weeks post-fertilization. Therefore, there appear to be two kinds of dividing cell in zebrafish brain. Neurogenesis in the telencephalon seems to be similar to higher vertebrate neurogenesis: replication was localized near the ventricle and ceased at an early stage. In contrast, neurogenesis in the margin of the tectum and the top of the cerebellum continued after maturation.

The difference between the two cell types is not readily apparent, but a recent report indicated that neural stem cells in higher vertebrates did not lose their replicative ability. Replication in these cells is strongly repressed, so that cell division is arrested in the tissue; however, in tissue culture, especially in the presence of neurotrophic factors such as basic FGF, the cells recover their replicative activity. Thus, it might be possible to restore replicative activity in the zebrafish telencephalon using such factors.

## ACKNOWLEDGMENTS

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