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Peripheral sensory deafferentation affects olfactory bulb neurogenesis in zebrafish

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Abstract

The potential effects of removal of olfactory input on adult neurogenesis in the olfactory bulb were examined. Olfactory organs of adult zebrafish were permanently and completely ablated by cautery and animals were exposed to bromodeoxyuridine then examined following short (4 hour) or long (3 week) survival periods. Short survival times allowed analysis of cell proliferation in the olfactory bulb. Long survival times permitted investigation of survival of adult-formed cells. Deafferentation did not immediately affect the dividing cells in the bulb but did affect the number of adult-formed cells, some of which expressed a neuronal marker, present in the bulb three weeks later. Thus, afferent removal influenced the fate of newly formed cells by impacting subsequent divisions, maturation, or survival of those cells. One week of deafferentation altered the pattern of cell genesis, with a significant increase in the number of dividing cells located in the olfactory bulb and also in the ventral telencephalic proliferation zone. Sham surgery did not impact either proliferation or survival of adult-formed cells in the olfactory bulb, suggesting that the deafferentation effect is specific. Thus, afferent innervation is necessary for normal cell proliferation and maintenance of the olfactory bulb in adult zebrafish.

Keywords

bromodeoxyuridine; plasticity; proliferation; ablation; denervation; teleost

1. INTRODUCTION

It has been known for many years that adult neurogenesis occurs constitutively in a few regions of the mammalian brain, specifically the olfactory bulb (Altman, 1969; Kaplan and Hinds, 1977; Bayer, 1983; Corotto et al., 1993) and the hippocampal dentate gyrus (Altman and Das, 1965; Bayer, 1982; Kaplan, 1984). The dentate gyrus is a site of proliferation and maturation of adult-formed hippocampal granule neurons (Gould et al., 1998; Cameron et al., 1993). The neurogenic region for the olfactory bulb is the subventricular zone of the lateral ventricles (Luskin, 1993; Lois and Alvarez-Buylla, 1993). Newly formed cells born in the subventricular zone migrate through the rostral migratory stream to the olfactory bulb, where they become interneurons (Lois and Alvarez-Buylla, 1993; Bédard and Parent, 2004; Zheng, et al., 2006). Although the number of newly added cells appears to be a small proportion of the total population present in the adult brain, this process does appear to be significant (Gross, 2000).

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The persistence of cell genesis in the mature brain is even more pronounced in animals such as frogs, reptiles, birds, crustaceans, and fish (reviewed in Lindsey and Tropepe, 2006). Fish, in particular, are prominent models in the field of adult neurogenesis due to their extensive neurogenic abilities (reviewed in Lindsey and Tropepe, 2006). While cell proliferation occurs in numerous brain regions of teleosts, it is especially noticeable in the cerebellum (Zupanc and Horschke, 1995), optic tectum (Nguyen et al., 1999), telencephalon (Alonso et al., 1989), and around the ventricles (Ekström et al., 2001). The zebrafish provides a good model for analysis of neurogenesis since this species is commonly studied and has been shown to possess several mitotic regions in the adult brain (Rahmann, 1968; Huang and Sato, 1998; Zupanc, 1999; Byrd and Brunjes, 2001; Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006; Hinsch and Zupanc, 2007). Most teleost species display indeterminate growth, where the body continues to increase throughout the lifespan. This reason is cited as an explanation for the robust neurogenesis that fish possess (Zupanc, 1999). However, zebrafish appear to reach a growth plateau at 4 cm and under 0.5 g (Gerhard et al., 2002; Biga and Goetz, 2006). Thus, persistent neurogenesis in zebrafish may not be as pronounced as in other teleosts and may be more similar to that in species such as mammals that have determinate growth.

The morphology of the zebrafish olfactory bulb is typical of teleosts (Byrd and Brunjes, 1995). The bulb is diffusely organized into three main laminae: the olfactory nerve, glomerular, and internal cell layers. The olfactory nerve layer consists of afferent axons from the olfactory epithelium intermingled with glial cells. The glomerular layer is the middle region that contains identifiable glomeruli where olfactory axon terminals interact with dendrites of bulb neurons including mitral cells and juxtaglomerular neurons (Baier and Korsching, 1994; Byrd and Brunjes, 1995; Edwards and Michel, 2002; Fuller, Yettaw, and Byrd, 2006). The internal cell layer is the inner core of the bulb containing numerous interneurons (Edwards and Michel, 2002). Because the zebrafish olfactory bulb is similar in structure to other animals, information from this fish may prove useful for understanding neurogenesis in other species.

One common method of examining adult brain plasticity is identifying changes that occur within a central structure following removal of afferent input. In both vertebrates and invertebrates, olfactory deafferentation causes changes in the central olfactory structures. In crayfish, unilateral antennular amputation decreases the volume of the olfactory lobe and the number of interneurons (Sandeman et al., 1998). In mammals, odor deprivation (Maruniak et al., 1989; Baker et al., 1993; Cho et al., 1996) and chemical lesioning of the olfactory epithelium (Harding et al., 1978; Baker et al., 1983) profoundly affects the neurochemistry and morphology of the olfactory bulb. In fish, peripheral sensory deafferentation in adults, by unilateral olfactory-organ ablation, has a significant effect on the size and morphology of the olfactory bulb (Byrd, 2000). These studies illustrate the trophic relationship between axons from the olfactory organ and their target in the brain in adult animals.

We examined how removal of primary afferent axons affects the generation and maturation of new cells in the adult brain. Previous studies on neurogenesis in the adult zebrafish have shown that while cell genesis within the olfactory bulb is not dramatic, it does occur and additional cells, some of which are neurons, are added to the olfactory bulb of adults (Byrd and Brunjes, 2001; Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006; Hinsch and Zupanc, 2007). Many of these adult-formed cells originate from the ventral telencephalic proliferation zone (Adolf et al., 2006; Grandel et al., 2006). In the current study we analyzed and compared the levels of cell genesis in surgically deafferented animals and control animals. Generation of new cells was monitored following denervation using the thymidine analog bromodeoxyuridine (BrdU) that was administered at several time points after olfactory-organ ablation. Experimental groups included animals surviving 4 hours after BrdU administration, to examine the effects on proliferation, and 3 weeks following exposure to the marker, to

analyze the fate of newly formed cells (Table 1). A significant effect of deafferentation on both cell proliferation and cell fate was observed.

2. RESULTS

Analysis of immunoreactivity for BrdU allowed identification of the nuclei of cells that had incorporated the thymidine analog during the S-phase of the cell cycle. Examination of sections from animals killed at the short survival time (4 hours after BrdU treatment) allowed investigation into effects on cell proliferation, and sections from animals killed at the long survival time (3 weeks after receiving the drug) allowed exploration of effects on cell differentiation. Unoperated control animals that were allowed to survive longer following BrdU administration possessed significantly more BrdU-positive profiles in their olfactory bulbs than those killed soon after exposure (Fig. 1A,C; $P = 0.01$), which is consistent with previous reports (Byrd and Brunjes, 2001) and likely represents either division of newly formed cells or migration of new cells into the bulb. Olfactory bulbs from unoperated control animals at the 4-hr survival time had BrdU-labeled profiles primarily in the outer bulb layers including the olfactory nerve and glomerular layers (Fig. 1A), while those from the 3-week survival time showed more profiles throughout the bulb including the olfactory nerve, glomerular, and internal cell layers (Fig. 1C). There was variability in the number of BrdU-positive profiles in the olfactory bulbs of the control groups (Fig. 2), although unoperated, control and sham-operated animals analyzed at either survival time post-BrdU administration showed no significant difference in the average numbers of BrdU profiles between right and left bulbs. Thus, there was no inherent difference between the two sides of the olfactory bulb in cell proliferation or cell fate.

Animals that underwent olfactory organ ablation and then were exposed to BrdU on the same day showed no significant difference in the number of cells that had incorporated BrdU when examined 4 hours after deafferentation (Fig. 3; $P = 0.37$). However, there was an effect on either subsequent divisions, maturation, or survival of these cells since there was a significant decrease in the total number of BrdU-positive cells on the operated side in the animals that were deafferented and exposed to BrdU the same day and then killed 3 weeks after receiving the drug (Fig. 3; $P = 0.01$). BrdU-immunoreactive nuclei were still found in deep bulb layers (Fig. 1D), but their numbers were obviously diminished.

Deafferentation had a significant effect on the number of proliferating cells in the right, deafferented olfactory bulbs one week following olfactory organ ablation (Fig. 1B). There was an increase in the total number of cells in S-phase during the availability of BrdU in the denervated olfactory bulbs of deafferented animals at short survival times following BrdU exposure compared to the intact side (Fig. 3; $P = 0.03$). This was especially apparent in the olfactory nerve layer, which had an average of 14.2 ± 2.2 profiles in the deafferented bulb and 10.8 ± 1.6 profiles in the intact bulb ($P = 0.04$). The left, untreated bulbs had no difference in number of BrdU-immunoreactive nuclei compared to unoperated controls and other deafferented groups ($P > 0.05$). The total number of BrdU-labeled nuclei three weeks after BrdU exposure is not significantly different from unoperated control levels (Fig. 3; $P = 0.74$) suggesting that the survival of the newly formed cells was not affected. However, there was an elevation in the number of BrdU profiles in the glomerular layer, with 4.3 ± 1.8 in the deafferented bulb and 1.3 ± 0.8 in the intact bulb ($P = 0.04$). Some of these newly formed cells are neurons since they co-labeled with the neuronal marker anti-HuC/HuD (Fig. 4).

Animals that were deafferented and allowed to live for three weeks before being exposed to BrdU showed no difference between the two bulbs in number of BrdU-labeled profiles at either 4 hours ($P = 0.91$) or 3 weeks ($P = 0.41$) post-BrdU administration (Fig. 3). The longer survival period allowed for the addition of more newly formed cells to the olfactory bulb. In fact, this

group had increased numbers of labeled profiles in both right, deafferented and left, intact bulbs compared to unoperated control animals, sham-operated animals, and animals deafferented for one week before BrdU exposure ($P < 0.05$). Even at these long survival times post-deafferentation (6 weeks for this group), there was no regrowth of olfactory axons with our permanent deafferentation method (Byrd, 2000).

Since the origin of many adult-formed cells that are added to the olfactory bulb of zebrafish is the proliferation zone around the ventral telencephalic ventricular region (Adolf et al., 2006; Grandel et al., 2006), this area was examined for potential effects of deafferentation. Four hours after BrdU exposure, there were numerous proliferating cells clustered around the ventricle of all groups examined, and there were fewer cells remaining in this brain region 3 weeks after the single exposure to BrdU (Fig. 5). A quantitative analysis of the density of BrdU-labeled cells revealed that there was a significant increase in the amount of proliferating cells around the ventricle on the experimental side (0.0078 ± 0.0003) compared to the intact side (0.0057 ± 0.0005) of animals that were deafferented for one week prior to exposure to BrdU (Fig. 5 D; $P = 0.04$). There were no other significant differences observed in ventricular zone density when right, deafferented and left, intact sides were compared or when right, deafferented sides were compared between groups ($P > 0.05$ for paired t-test and ANOVA comparisons).

3. DISCUSSION

Our analysis was performed on fish that were exposed to BrdU by immersion in a solution of the drug for one hour, with uptake presumably via the gills. BrdU remains metabolically active for approximately 30 minutes (Hinsch and Zupanc, 2007) to four hours (Zupanc and Horschke, 1995) after administration via intraperitoneal injection. Thus, our study investigated the number of cells that were in the S-phase of mitosis during the one hour exposure period and up to four additional hours immediately following. Immunocytochemistry allowed visualization of newly formed cells. For our long-term survival period of 3 weeks, it is unclear if the cells divided only once since BrdU exposure or multiple times, as has been reported in other studies (Zupanc et al., 2005). Also, it is possible that some cells that picked up the drug died or divided so many times that the BrdU was diluted to the point of not being visualized with the antibody. Our results were analyzed with these experimental limitations in mind.

We found that there is a decrease in the number of adult-formed cells found in the olfactory bulb 3 weeks after removal of sensory input. This reduction does not appear to be due to fewer cells picking up BrdU in the hours following olfactory-organ ablation because animals that were deafferented, treated with BrdU on the same day, and killed 4 hours later showed no difference from unoperated controls in the number of dividing cells. Thus, the effect appears to be on cell differentiation, maturation, or survival. Similarly, olfactory nerve transection in late-stage larval frogs does not appear to influence precursor cell division in the ventricular zone, but this manipulation does affect mitral cell maturation or survival (Burd and Sein, 1998). It is possible that in our analysis fewer cells matured or that there were fewer divisions of cells due to apoptosis following deafferentation that killed the precursor cells. Indeed, a previous study from our lab examined apoptosis in response to the same form of deafferentation (VanKirk and Byrd, 2003). There is a significant increase in number of apoptotic cells in the olfactory bulb peaking one hour and 24 hours following removal of the olfactory organ. Thus, the process of cell production may proceed normally immediately following peripheral deafferentation, but the survival of the newly formed cells may have been prevented by the lack of afferent input.

Another major finding in this study is that there are increased numbers of newly generated cells in both the bulb and the telencephalic proliferation zone one week following removal of sensory input. This is especially apparent in the olfactory nerve layer of the bulb, which consists

primarily of the axons of olfactory sensory neurons and non-neuronal cells (Byrd and Brunjes, 1995). The proliferation of newly formed cells in this region likely represents the increased division of non-neuronal cells. Perhaps this is a response to the degenerating axons of the severed olfactory nerve. This bulb layer is diminished by one week following ablation of the olfactory organ, is absent by three weeks following deafferentation, and does not return in our permanent deafferentation method (Byrd, 2000). The time course of nerve degeneration and likely gradual remodeling in that area is consistent with our findings of increased cell division. It is possible that the proliferation of cells in the olfactory bulb observed one week following deafferentation is part of a reorganization of the bulb in response to damage. This increase appears to be transient, since most of those cells do not remain in the bulb three weeks later. It is interesting that this time point is also when we found an effect on proliferation of cells around the ventral telencephalic ventricle. When animals are deafferented and then exposed to BrdU one week later, there is a significant increase in proliferation on the experimental side compared to the intact side. A possible explanation is that cell proliferation is upregulated one week after deafferentation, but that many of those newly formed cells do not survive. A similar study in mammals supports this idea. Mice were subjected to deafferentation via severing the olfactory sensory axons and eight days later they were injected with BrdU and killed two hours later (Mandairon et al., 2003). There was a bilateral increase in proliferation of cells with the greatest effect observed in the extension of the rostral migratory stream within the olfactory bulb, and this was countered by increased cell death on the operated side.

Fish that were allowed to survive three weeks following deafferentation before BrdU administration showed no difference in cell proliferation from control levels. By this time, the nerve has degenerated and axonal debris been cleared away, so no additional cells may be needed for remodeling of the bulb. Those animals that survived three additional weeks following BrdU have no difference in surviving cells from the intact side, but there are more newly formed cells surviving in both bulbs compared to unoperated control animals, sham-operated animals, and those fish deafferented and treated with BrdU on the same day. It is unclear why this group showed an increased survival, further mitosis, or effects on maturation of newly formed cells. A similar effect was noted following olfactory denervation in mice where a bilateral increase in cell proliferation in the rostral migratory stream and olfactory bulb was observed (Mandairon et al., 2003). In decapod crustaceans, Hansen and Schmidt (2001) found that unilateral antennule amputation affected the entire olfactory system, since the contralateral olfactory lobe showed a more substantial decrease in cell proliferation than even the ipsilateral side. Although these studies showed opposite effects of afferent removal, both showed alterations in the pattern of cell genesis both ipsilateral and contralateral to the experimental side similar to our findings.

There are many other examples of olfactory deprivation affecting neurogenesis in the adult brain of many other animals. Naris occlusion in adult mice causes reduced neurogenesis in the ipsilateral olfactory bulb (Corotto et al., 1994). Amputation of antennules in the adult shore crab reduces the rate of proliferation of neuronal precursor cells and impacts the survival of newly formed cells in the olfactory lobes (Hansen and Schmidt, 2001). In crickets, unilateral olfactory deprivation inhibits central neurogenesis, when performed with visual deprivation (Scotto-Lomassese et al., 2002). Olfactory axotomy increases cell genesis in the subventricular zone, rostral migratory stream, and olfactory bulb (Mandairon et al., 2003). Survival of adult-formed neurons, however, is decreased without olfactory activity (Corotto et al., 1994; Petreanu and Alvarez-Buylla, 2002; Mandairon et al., 2003). Thus, sensory input supports the survival of newly formed cells in the adult bulb. There are other studies, however, that found no effect of afferent input on cell genesis. Anosmic mice continue to have normal levels of production, migration, and differentiation of adult-born granule cells (Petreanu and Alvarez-Buylla, 2002).

In most mammals, adult-generated cells born in the subventricular zone migrate through the rostral migratory stream to the olfactory bulb, where they become interneurons (Lois and Alvarez-Buylla, 1993; Bédard and Parent, 2004; Zheng, et al., 2006). It appears that a similar process is utilized in zebrafish. Our previous study showed that the number of newly generated cells in the olfactory bulb of adult zebrafish increases between 4 hours and 4 weeks following BrdU exposure (Byrd and Brunjes, 2001). We reasoned that this increase could be due to either a rostral migratory stream-equivalent in zebrafish or proliferation of cells within the bulb. An elegant series of experiments by Grandel and colleagues (2006) attempted to distinguish between these alternatives using pulse-chase labeling with three proliferation markers: bromodeoxyuridine, iododeoxyuridine, and anti-proliferating cell nuclear antigen. They found evidence of a minor rostral migratory stream and suggested that additional migratory routes were also present in zebrafish. Newly formed cells can be observed in the olfactory bulb as soon as 3 days following BrdU administration but are numerous after 2 weeks (Adolf et al., 2006). The origin of these neurons appears to be the progenitors located at the ventricle of the ventral telencephalon. In fact, a stripe of a specific cell-surface glycoprotein antibody labeling was observed reaching from the subpallial region to the olfactory bulb (Adolf et al., 2006) comparable to the organization of the rostral migratory stream in mammals (Doetsch et al., 1997). Thus, the addition of new cells to the zebrafish olfactory bulb likely occurs by cells born in the telencephalon and migrating into the bulb. The current study lends support to this idea since we found that there are more BrdU-positive cells around the ventral telencephalic ventricle at 4 hours after BrdU exposure compared to 3 weeks survival. The diminution of BrdU labeling with longer survival times could be due to dilution of the thymidine analog with repeated division of cells that picked up the drug during the brief exposure or migration of BrdU-positive cells away from the ventricular region.

Many newly formed cells in the zebrafish olfactory bulb differentiate into neurons, as shown by anti-HuC/HuD labeling (Byrd and Brunjes, 2001; Grandel et al., 2006). In mammals, new neurons that are added to the adult olfactory bulb differentiate into GABAergic granule and dopaminergic periglomerular cells (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Similarly, some newly formed neurons in the adult zebrafish olfactory bulb express a GABA synthesizing enzyme Gad1 (Adolf et al., 2006). Fewer of these cells, however, become catecholaminergic interneurons (Grandel et al., 2006). Thus, adult neurogenesis in zebrafish is similar to mammals and generates at least two types of neurons in the olfactory bulb: GABAergic and dopaminergic interneurons.

Our sham operation was designed to control for the potential influence of wounding near the olfactory bulbs. We found no effects of the sham surgery on incorporation or survival of BrdU. Thus, it is unlikely that a general wound response from the skin causes fewer cells to divide or mature. This supports the idea that the effects reported in this study are in response to removal of afferent input to the brain.

Deprivation of olfactory input influences cell genesis in the olfactory bulb and ventral telencephalic ventricular region of adult zebrafish. Whether the influence is due to contact by olfactory axons or activity from the olfactory organ is unclear and requires further investigation. The mechanisms that regulate persistent neurogenesis in the adult brain are the topic of intensive investigation (Hagg, 2005; Ming and Song, 2005). Many external and internal cues have been examined for their potential influence on the rate of cell proliferation in the adult brain (reviewed in Lindsey and Tropepe, 2006). Future studies will attempt to elucidate the mechanisms by which olfactory axons exert an influence on the process of adult neurogenesis.

4. EXPERIMENTAL PROCEDURES

Adult zebrafish were purchased from a local commercial supplier and maintained in 10-gallon aquaria at 28°C. Approximately 75 fish of both sexes, ranging in size from 2.5 to 4.0 cm and all over 4 months of age, were used in this study. Zebrafish are considered adult at the onset of fecundity, generally at 3 months of age when reared at 28°C (Kimmel, 1989). All efforts were made to minimize animal suffering and the number of animals used. All procedures were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Deafferentation Procedure

Thirty-nine adult zebrafish were anesthetized with 0.03% MS222 (ethyl 3-aminobenzoate methanesulfonate salt, Sigma), and the right olfactory organ was ablated using a small-vessel cautery iron as described previously (Byrd, 2000). The left olfactory organ was left undamaged for use as an internal control. Unoperated control fish (n=6) were anesthetized in the same manner but received no cautery wound. Sham-operated fish (n=14) received a wound with the cautery iron to the skin between the olfactory organs. Fish were returned to aquarium water containing the antibiotic kanamycin and were treated with bromodeoxyuridine on the same day as surgery or 1 or 3 weeks following surgery (Table 1).

Identification of Newly Generated Cells

Cell genesis was monitored using the thymidine analog, bromodeoxyuridine (BrdU). BrdU treatment and immunocytochemistry were performed as described previously (Byrd and Brunjes, 2001). Deafferented, sham-operated, and unoperated control fish were exposed to a 1% solution of 5-bromo-2'-deoxyuridine (Sigma) for one hour in a small aquarium to allow for uptake of the thymidine analog via the gills. Fish were returned to aquaria containing tank water that was changed every hour for two hours, to minimize reuptake of excreted BrdU. After the appropriate survival period of 4 hours or 3 weeks, the fish were processed for BrdU immunocytochemistry.

Animals were over-anesthetized in 0.03% MS222 and perfused transcardially with phosphate buffered saline followed by Bouin's fixative solution. The brains were dissected after 2 hours in fixative and embedded in paraffin following typical protocols, and 10-µm horizontal sections were mounted onto gelatin-coated or positively charged slides. Slides were dewaxed, rehydrated, and reacted for 10 minutes with 3% hydrogen peroxide to inactivate endogenous peroxidases. Following a buffer rinse, slides were incubated with 2N hydrochloric acid for 20 minutes at 37°C and neutralized with two 5-minute rinses in borate buffer. Slides were immersed in 0.1M phosphate buffer with 3% normal goat serum and 0.4% Triton X-100 for one hour to minimize non-specific staining and then incubated for 20 hours at 4°C in a monoclonal antibody to BrdU (Dako) diluted 1:100 with the blocking solution. Slides were washed with phosphate buffer and immersed in biotinylated goat anti-mouse secondary antibody (Dako) diluted 1:100 in blocking solution for one hour at room temperature. Following a buffer rinse, slides were placed in an avidin-biotin peroxidase solution (ABC Vectastain elite, Vector Laboratories) for 90 minutes and reacted with 3, 3'-diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories) for 2-10 minutes. After rinsing in buffer, dehydrating with alcohols, and clearing with xylenes, slides were coverslipped with DPX (Aldrich). Negative controls, in which the primary antibodies were deleted, consistently revealed no staining.

Quantification of Newly Formed Cells

Quantitative analyses were performed using the methods of unbiased stereology on semi-serial sections from 3-9 animals for each survival group. An estimate of the average number of BrdU-

labeled cells in each olfactory bulb was made by counting the number of labeled profiles in every 6th section and summing the number of profiles counted in the right and left bulbs. To examine the distribution of labeled nuclei, the location of BrdU-positive profiles was separated into the three layers of the olfactory bulb: the olfactory nerve, glomerular, and internal cell layers. To examine cell proliferation in the ventral telencephalic ventricular zone, the density of BrdU-positive profiles in this area was quantified. Area measurements and counts of BrdU-labeled profiles were obtained from two sections that were 50 μ m apart for three animals from each group. The density of BrdU labeling was calculated by averaging the densities in the two sections from each animal. For all quantitative analyses, the entire 10- μ m thickness was viewed by focusing through the section. The lightness or darkness of the immunostaining was not differentiated, meaning that some BrdU-positive profiles may have divided numerous times during the survival period. Average values for all analyses were reported with the standard error of the mean (average $\pm$ SEM). Percent difference between right and left bulbs was calculated for each group as (average of right bulb – average of left bulb / average of right bulb) $\times$ 100. Statistical determinations of differences between right, deafferented and left, intact olfactory bulbs were based on the paired-sample t-test. Statistical comparisons between groups were based on the t-test or analysis of variance with the Tukey test for multiple comparisons. A significance level of 0.05 was used.

Identification of Newly Formed Neurons

Brains of BrdU-treated animals from various survival groups were over-anesthetized, perfused, processed, and sectioned as above. Slides were dewaxed, rehydrated, incubated with 2N hydrochloric acid for 20 minutes at 37°C, neutralized with two 5-minute rinses in borate buffer, and then subjected to antigen retrieval with 10 minutes in 10mM sodium citrate at 100°C. Slides were immersed in 0.1M phosphate buffer with 3% normal goat serum and 0.4% Triton X- 100 for one hour to minimize non-specific staining and then incubated for 20 hours at 4°C in a monoclonal antibody to BrdU (Dako) diluted 1:100 with the blocking solution. Slides were washed with phosphate buffer and immersed in biotinylated goat anti-mouse secondary antibody (Dako) diluted 1:100 in blocking solution for one hour at room temperature. Following a buffer rinse, slides were placed in AlexaFluor 568 avidin (Molecular Probes) diluted 1:1000 in PBS for one hour at room temperature. Subsequent treatment of slides was performed while protecting them from light. Slides were rinsed for 24 hours in several changes of PBS then incubated for 20 hours at 4°C in monoclonal anti-HuC/HuD (Molecular Probes) diluted 1:100 in the blocking solution. Following PBS rinses, slides were incubated in AlexaFluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) diluted 1:100 in PBS for one hour at room temperature. Slides were rinsed and coverslipped with glycerol containing para-phenylenediamine (Sigma). Negative controls, in which the primary antibodies were deleted, consistently revealed no staining.

All photomicrographs were collected with a digital camera. Manipulations were made only to brightness, contrast, or color levels using Adobe Photoshop 6.0 (Adobe Systems).

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Figure 1.

Examination of cell genesis in the olfactory bulbs of unoperated control (unop) and deafferented (deaff) adult zebrafish. Anti-bromodeoxyuridine (BrdU) labeling of right olfactory bulbs from unoperated control (A, C) and deafferented (B, D) animals allowed to survive 4 hours (A, B) or 3 weeks (C, D) following administration of BrdU. Short-term survival animals with intact sensory input had several BrdU-positive nuclei in the olfactory nerve layer (A), and long-term survival control animals had numerous BrdU-labeled cells in all bulb layers (C). Animals that were exposed to BrdU one week after peripheral deafferentation had many more BrdU-positive nuclei throughout the bulb layers (B) when examined four hours after BrdU exposure. Animals that were deafferented and exposed to BrdU on the same day showed

an obvious reduction in BrdU-labeled nuclei when examined three weeks later (D). Arrows indicate immunoreactive profiles. Scale bar = 50 μ m for all.

Figure 2.

Control groups show no inherent difference in cell genesis or fate between right and left bulbs. The number of anti-bromodeoxyuridine-labeled profiles in olfactory bulbs of unoperated (unop) and sham-operated (sham) control animal groups was compared using mean percent difference ($\pm$ SEM) between right and left sides. Animals examined 4 hours after BrdU incorporation (short-term survival) and 3 weeks following BrdU exposure (long-term survival) showed no significant difference in BrdU counts between right and left olfactory bulbs.

Figure 3.

Effects of deafferentation on cell proliferation and maturation in the olfactory bulbs. Bromodeoxyuridine-positive profiles were counted in semi-serial sections through the entire olfactory bulbs of unoperated control and deafferented animals from 4 hour (short-term) survival and 3 week (long-term) groups. Right, deafferented and left, intact sides were compared for each group. At most times examined, there was no significant difference between the two sides of the olfactory bulb. One week following denervation (1w Deaff), there were more BrdU-positive cells in the deafferented bulb compared to the internal control bulb. Animals that were deafferented and treated with BrdU on the same day (0d Deaff) had fewer

BrdU+ profiles in the bulb three weeks later. Asterisk indicates a significant difference using paired-sample t-test ($P < 0.05$).

Figure 4. Some cells born one week following deafferentation and allowed three weeks to mature became neurons. Immunofluorescence of the glomerular layer of the olfactory bulb shows bromodeoxyuridine-positive profiles (A), the neuronal marker anti-HuC/HuD (B), and cells that co-labeled (C). Scale bar = 10 μ m.

Figure 5.

Deafferentation affects cell genesis in the ventral telencephalic proliferation zone. A) Unoperated control animals (unop) have numerous bromodeoxyuridine-labeled profiles surrounding the ventricle (V) 4 hours after BrdU administration. B) One week after deafferentation, BrdU-immunoreactive nuclei are more numerous. C) Fewer cells are present around the ventricles of unoperated control animals 3 weeks after exposure to BrdU, likely due to either dilution following multiple cell divisions or migration of cells away from the ventricle. D) A comparison of the density of BrdU-positive cells in unoperated control and deafferented animals examined after short-term (4 hours) survival reveals a significant difference in density of the deafferented bulb compared to the internal control bulb of animals that had BrdU administered one week following deafferentation. No significant differences between right, deafferented and left, intact sides are found in the long-term (3 weeks) survival analysis. Asterisk indicates $P < 0.05$. V = telencephalic ventricle. Scale bar = 20 μ m.

Table 1

Animal Treatment Groups Examined in This Study¹

Group	Surgery	Post-surgery period before BrdU exposure	Post-BrdU survival	Total time deaff.	N
<u>Short-term Survival:</u>					
Unop.	None		4 hours	0	3
1w Sham	Sham	1 week	4 hours	0	4
0d Deaff.	Deafferented	2 hours	4 hours	0	8
1w Deaff.	Deafferented	1 week	4 hours	1 week	9
3w Deaff.	Deafferented	3 weeks	4 hours	3 week	6
<u>Long-term Survival:</u>					
Unop.	None		3 weeks	0	3
0d Sham	Sham	2 hours	3 weeks	0	4
3w Sham	Sham	3 weeks	3 weeks	0	6
0d Deaff.	Deafferented	2 hours	3 weeks	3 weeks	5
1w Deaff.	Deafferented	1 week	3 weeks	4 weeks	6
3w Deaff.	Deafferented	3 weeks	3 weeks	6 weeks	5

¹The group names denote treatment and survival time before BrdU exposure. The numbers of animals used, surgical procedures administered, survival times following surgery and BrdU exposure, and total amount of time the fish were deafferented before sacrifice are described. BrdU exposure followed the post-surgery survival time, and the animals were fixed after the post-BrdU survival period.