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Patterns of Olfactory Bulb Neurogenesis in the Adult Zebrafish are Altered Following Reversible Deafferentation

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Abstract

Adult brain plasticity can be investigated using reversible methods that remove afferent innervation but allow return of sensory input. Repeated intranasal irrigation with Triton X-100 in adult zebrafish diminishes innervation to the olfactory bulb, resulting in a number of alterations in bulb structure and function, and cessation of the treatment allows for reinnervation and recovery. Using bromodeoxyuridine, Hu, and caspase-3 immunoreactivity we examined cell proliferation, differentiation, migration, and survival under conditions of acute and chronic deafferentation and reafferentation. Cell proliferation within the olfactory bulb was not influenced by acute or chronic deafferentation or reafferentation, but cell fate (including differentiation, migration, and/or survival of newly formed cells) was affected. We found that chronic deafferentation caused a bilateral increase in the number of newly formed cells that migrated into the bulb, although the amount of cell death of these new cells was significantly increased compared to untreated fish. Reafferentation also increased the number of newly formed cells migrating into both bulbs, suggesting that the deafferentation effect on cell fate was maintained. Reafferentation resulted in a decrease in newly formed cells that became neurons and, although death of newly formed cells was not altered from control levels, survival was reduced in relation to that seen in chronically deafferented fish. The potential effect of age on cell genesis was also examined. While the amount of cell migration into the olfactory bulbs was not affected by fish age, more of the newly formed cells became neurons in older fish. Younger fish displayed more cell death under conditions of chronic deafferentation. In sum, our results show that reversible deafferentation affects several aspects of cell fate, including cell differentiation, migration, and survival, and age of the fish influences the response to deafferentation.

Keywords

Teleost; Bromodeoxyuridine; Neurogenesis; Olfactory bulb; Deafferentation; Reafferentation

INTRODUCTION

Adult neurogenesis in the vertebrate brain has become widely recognized in the past two decades, with the olfactory system emerging as an excellent model system for studies

investigating adult brain plasticity. The olfactory system is easily accessible, has well-documented morphology and circuitry, and has an innate, persistent adult neurogenic capacity in the peripheral olfactory epithelium (Moulton et al., 1970; Graziadei and Graziadei, 1979) and the central olfactory bulb (Altman, 1969; Kaplan and Hinds, 1977; Bayer, 1983; Corotto et al., 1993; Adolf et al., 2006; Grandel et al., 2006).

In the adult mammalian brain, the subgranular zone of the dentate gyrus (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006) in the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977) and the subventricular zone of the lateral wall of the lateral ventricles (Altman, 1969; Kaplan and Hinds, 1977) are the two regions of constitutive neurogenesis. Stem cells in the subgranular zone generate neuroblasts that mature into granule cells of the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Altman and Bayer, 1990). In the subventricular zone, neural stem cells generate neural precursor cells (Lois and Alvarez-Buylla, 1993; Luskin, 1993) that migrate through the rostral migratory stream into the olfactory bulb (Lois and Alvarez-Buylla, 1994; Luskin, 1993; Rousselot et al., 1995; Doetsch and Alvarez-Buylla, 1996; Jankovski and Sotelo, 1996). Once the neural precursor cells reach the olfactory bulb, most mature into granule and periglomerular interneurons (Lois and Alvarez-Buylla, 1994; Luskin, 1993; Betarbet et al., 1996; Winner et al., 2002).

The adult zebrafish brain displays more abundant adult neurogenesis, with 16 distinct neurogenic niches that are distributed along the entire rostro-caudal brain axis (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006). Two of these are equivalent to the mammalian subventricular zone and subgranular zone: the telencephalic ventricular zone and dorsolateral domain, respectively (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006). The zebrafish telencephalic ventricular zone maintains a population of neural stem cells that generate neural precursor cells characteristically similar to those generated in the mammalian subventricular zone (Zupanc et al., 2005; Lam et al., 2009; März et al., 2010; Kishimoto et al., 2011). These cells migrate through a rostral migratory stream to the olfactory bulb and differentiate into mature interneurons (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006; Kishimoto et al., 2011).

One of the additional proliferating domains identified in adult zebrafish is the olfactory bulb (Zupanc et al., 2005; Grandel et al., 2006). Proliferating cells are found scattered throughout the 3 diffuse concentric layers of the adult zebrafish olfactory bulb (Byrd and Brunjes, 2001; Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006): the outermost olfactory nerve (ONL), the middle glomerular (GL), and the inner internal cellular (ICL) layers.

Approximately half of the newly generated cells in the bulb express a neuronal identity (Zupanc et al., 2005; Adolf et al., 2006). Nevertheless, the number of proliferating cells is so meager that it is not considered a major source of newly generated cells (Zupanc et al., 2005; Grandel et al., 2006). Thus, similar to mammals, the addition of newly generated cells to the adult zebrafish olfactory bulb consists primarily of the migration of cells into the bulb from the telencephalic ventricular zone and not intrinsic bulbar cell genesis.

The effect of afferent input on the adult olfactory bulb, while not fully elucidated, has been shown to be crucial for homeostatic maintenance of this brain region, and sensory

deprivation has been shown to be deleterious. Sensory deprivation has been achieved through various deafferentation methods, including reversible, unilateral naris occlusion. In juvenile rats, the reduction in bulb volume and tyrosine hydroxylase expression seen with naris occlusion is reversed with the restoration of sensory input (Cummings et al., 1997). An alternative model of reversible deafferentation involves chemical ablation of the olfactory epithelium with a single application of Triton X-100, ZnSO₄, or methyl bromide. These lesions also result in a reduction in olfactory bulb weight and volume (Margolis et al., 1974; Harding et al., 1978; Schwob et al., 1999) and a decrease in tyrosine hydroxylase expression (Nadi et al., 1981; Baker et al., 1983). Recovery of the mammalian olfactory epithelium is substantial but incomplete at 30 days post lesion, with mature neurons reaching near control numbers at 4–6 weeks (Schwob et al., 1995; Cummings et al., 2000; Herzog and Otto, 1999). Thus, deafferentation through these methods allows investigation into short- and long-term effects on the olfactory bulb and its recovery.

In the adult zebrafish, reducing afferent input has similar effects on the olfactory bulb. Previous study in our lab has demonstrated that intranasal irrigation with Triton X-100 detergent produces an immediate degenerative effect on the olfactory epithelium followed by rapid regeneration of olfactory sensory neurons within five days (Iqbal and Byrd-Jacobs, 2010). A permanent, complete deafferentation method had shown previously that significant deafferentation-induced morphological alteration of the olfactory bulb occurred only following several weeks of deafferentation (Byrd, 2000). Thus, our lab developed a novel method for investigating the effects of long-term deafferentation and reafferentation of the olfactory bulb using repeated intranasal irrigation of detergent. This procedure results in reduction of olfactory bulb volume and tyrosine hydroxylase expression, while cessation of intranasal irrigation results in restoration of the olfactory epithelium, reinnervation of the olfactory bulb, and recovery of bulb volume and tyrosine hydroxylase expression (Paskin et al., 2011). Part of this loss of olfactory bulb volume is likely due to increased cell death since complete ablation of the olfactory organ by cautery results in a rapid, substantial increase in cell death (Vankirk and Byrd, 2003). Further, with this same manipulation, a gradual reduction in total cell number in the bulb occurs over several weeks (Byrd, 2000). Cell genesis does not appear to play a major role in this volume reduction following cautery since cell proliferation increased after this manipulation, although fate was affected (Villanueva and Byrd-Jacobs, 2009).

In the present study we used reversible deafferentation. Further, we investigate the possibility that cell genesis accounts, at least in part, for the deafferentation-induced reduction of olfactory bulb volume and reafferentation-induced recovery of bulb volume. We hypothesized that the diminished olfactory bulb volume seen following reduced afferent input is due to decreased cell genesis or reduced migration of newly formed cells into the olfactory bulb and that cessation of intranasal irrigation will result in the reversal of olfactory bulb volume reduction through increased cell genesis or enhanced migration of cells into the olfactory bulb.

EXPERIMENTAL PROCEDURES

A commercial vendor was used to obtain adult male and female zebrafish, *Danio rerio*, all of which were aged 5–9 months and approximately 3–4 cm in length. Additional adult male and female zebrafish, age 12–15 months, were a gift from Dr. Don Kane's lab. Fish were maintained in 28.5 °C, aerated, conditioned freshwater tanks with a 14h light: 10 h dark cycle and fed flake food twice daily at 7am-9am and 3pm-5pm. Intranasal infusions and intraperitoneal injections were performed between 7am-10am. Western Michigan University's Animal Care and Use Committee approved all experimental procedures. Precautions were taken to minimize the number and suffering of fish.

Chemical lesioning of the olfactory epithelium

Chemical lesioning was achieved by employing the protocol described previously (Iqbal and Byrd-Jacobs, 2010; Paskin et al., 2011). Briefly, zebrafish were anesthetized with MS222 (0.03%; Sigma, St. Louis, MO, USA) until they no longer responded to tail pinch and approximately 1 µL of a 0.7% Triton X-100 and 0.005% Methylene Blue solution prepared in 0.1M phosphate-buffered saline (PBS) was applied to the right nasal cavity. The untreated left side was used as an internal control. Following the 10 seconds it takes for intranasal infusion of the detergent, the fish were maintained on ice for 2 minutes out of water to prevent dilution of the detergent solution and ensure sufficient exposure as well as to maintain an appropriate level of anesthetization. The fish were then returned to their aquarium. In 3-week treated fish (chronic deafferentation group) this treatment was repeated every 72h (+/- 2h), while other fish received a single detergent application (acute deafferentation group).

Bromodeoxyuridine Intraperitoneal Injection Delivery

Anesthetized fish received an intraperitoneal injection along the ventral midline between the pelvic fins, using a nanofil 100 µl syringe and 35-gauge beveled needle (World Precision Instruments, Sarasota, FL, USA) to deliver 50 µL/g body weight of 20mM 5-bromo-2'-deoxyuridine (BrdU; Sigma) prepared in Dulbecco's PBS. Fish were then returned to their aquarium for survival periods of 4h (to examine proliferation) or 3 weeks (to determine cell fate).

Bromodeoxyuridine Immersion Delivery

Immersion delivery of BrdU was achieved by employing the protocol described previously (Byrd and Brunjes, 2001). Briefly, fish were exposed to a 1% solution of BrdU in a small aquarium for 1 h and then transferred to another small aquarium with water changes every hour for 4h before being returned to their aquarium for survival periods of 4h or 3 weeks.

Tissue Processing

All fish were over-anesthetized with 0.03% MS222 (Sigma) until opercular movement ceased, perfused with PBS, and fixed with 2% paraformaldehyde in PBS for 2h at room temperature or 24h at 4 °C. The brains were dissected, dehydrated in an ascending ethanol and xylene series, and embedded in paraffin for sectioning. Ten-µm coronal serial sections were mounted onto silanized slides and left at 37 °C overnight.

Immunocytochemistry and Microscopy

Mounted sections were dewaxed and rehydrated before undergoing antigen retrieval for 15 min in 10 mM sodium citrate solution at 96°C. Sections were rinsed in PBS and placed in blocking solution (3% normal goat serum, 1% bovine serum albumin, 1% fish gelatin, and 0.3% Triton X-100 in PBS) for 1h at room temperature and incubated for 48h at 4 °C in primary antibody diluted in blocking solution. Primary antibodies were anti-BrdU (newly formed cells; 1:200, Dako, Carpinteria, CA, USA), anti-keyhole limpet hemocyanin (KLH; olfactory axons; 1:1000, Sigma), anti-caspase-3 (apoptosis; 1:100, BD Biosciences, San Jose, CA), and anti-Hu (neuronal fate; 1:100, Thermo Fisher Scientific, Grand Island, NY, USA). Sections were rinsed in PBS and incubated with the appropriate secondary antibodies (Alexa Fluor 488-or 568-labeled goat anti-mouse or goat anti-rabbit (1:100, Thermo Fisher Scientific) for 1h at room temperature. For the BrdU and Hu double label, sections were processed as above, then placed in the second primary antibody prior to incubation in biotinylated goat anti-mouse secondary (1:200, Vector Laboratories, Burlingame, CA, USA) and Alex Fluor 568 streptavidin (1:100, Thermo Fisher Scientific). Finally, sections were rinsed in PBS and coverslipped with a polyvinyl alcohol-based aqueous, hardening mounting medium. Those sections processed for diaminobenzidine labeling followed the above protocol with the following exceptions: they were reacted briefly with 3% H₂O₂ to remove endogenous peroxidases, the primary antibody incubation was overnight at 4 °C, secondary antibody incubation was followed by immersion in avidin-biotin-peroxidase solution (ABC Vectastain Elite, Vector Laboratories) and reaction with diaminobenzidine, and sections were dehydrated in an ascending ethanol and xylene series before coverslipping with DPX mounting medium (Aldrich, St. Louis, MO).

For diaminobenzidine observations, stained sections were visualized with a Nikon Eclipse 80i microscope. For laser scanning confocal microscopy, fluorescently labeled sections were visualized using a Nikon C2 microscope. Manipulations to photomicrographs were made only to brightness, contrast and/or color levels using Nikon Elements or Adobe Photoshop CS3.

Quantification of Antibody-labeled Cells

Quantitative analyses were performed on serial sections from 3 fish for each control and treatment group. An estimate of the average number of BrdU-labeled profiles in the left and right olfactory bulbs was obtained by counting the number of labeled profiles in every fourth, 10µm section photographed as stacks of 10 1-µm thick optical sections at 60x and summing them. The location of the newly formed cells was obtained by noting the layer (ONL, GL, ICL) where the labeled profile was positioned. Counts of BrdU+/Hu+ and BrdU +/Caspase-3+ co-localized cells were similarly obtained from the left and right olfactory bulbs. To estimate the total volume of the olfactory bulb, the mean area of each semi-serial olfactory bulb section was determined using ImageJ (NIH). Area measurement from adjacent bulb sections were multiplied by 40 to account for the intervening sections and summed to estimate the volume for each bulb.

All statistical determinations were based upon mixed analysis of variance with a Bonferroni correction, using a significance level of 0.05.

RESULTS

BrdU was delivered to adult zebrafish employing both intraperitoneal injection and immersion methods in a pilot study to compare the efficacy of the two protocols in detecting proliferating cells in the adult zebrafish olfactory bulbs. Four hours after exposure to the drug by immersion, the number of BrdU-positive profiles in the olfactory bulbs was 12.3 ± 2.7 . With intraperitoneal injection there were 11.0 ± 3.5 BrdU-labeled cells in the bulbs. Since no significant differences were found in uptake of the drug between the two methods, but fish survival was superior (~50% survival for immersion and ~95% survival for injection) and reagent cost was reduced with the intraperitoneal injection method, this was the protocol chosen for the study.

When fish were given a 4h survival period following intraperitoneal injection of BrdU, there were few newly labeled cells in the 2 olfactory bulbs, with 12.3 ± 2.7 BrdU-positive profiles in both bulbs. Three weeks after BrdU delivery via intraperitoneal injection, there were more BrdU-positive profiles observed in the 2 olfactory bulbs, with 29.6 ± 8.3 labeled profiles in both bulbs. The short-term survival indicated that there was little intrinsic cell genesis in the olfactory bulbs, while the longer survival showed an increase in the number of newly formed cells over time, likely due to cell movement from outside of the bulb. These data are consistent with previous reports (Byrd and Brunjes, 2001; Zupanc et al., 2005; Grandel et al., 2006; Villanueva and Byrd-Jacobs, 2009).

The potential effects of deafferentation on cell proliferation in the olfactory bulbs were investigated by counting BrdU-positive profiles 4h following BrdU administration (Fig. 1A). Acute deafferentation was achieved with a single chemical ablation of the olfactory epithelium with Triton X-100 and chronic deafferentation resulted from repeated chemical ablation with the detergent for every 3 days over a 3-week period. In untreated control fish, acutely deafferented fish, and chronically deafferented fish, few BrdU-positive profiles were found in the olfactory bulbs in any group (Fig. 1B). No significant differences were found within or between groups. As others have reported, the limited BrdU-positive profiles observed in the olfactory bulbs at this time point were located primarily in the ONL, whereas each side of the ventral telencephalic ventricular zone was replete with BrdU-positive profiles (Byrd and Brunjes, 2001; Villanueva and Byrd-Jacobs, 2009). Anti-KLH was used to label olfactory sensory neuron axons the bulb (Fig. 1C). Even though afferents were still apparent in coronal sections of the deafferented bulb, innervation was extensively diminished on the medial side and substantially diminished on the lateral side (Fig. 1C). These findings are similar to the innervation patterns described in the deafferented bulb when examined in the transverse plane (Paskin et al., 2012) and in whole mounts (White et al., 2015). Additionally, the deafferented olfactory bulb volume ($0.12 \pm 0.02 \mu\text{m}^3$) was significantly reduced relative to the internal control bulb ($0.17 \pm 0.03 \mu\text{m}^3$), a finding similar to that described previously by Paskin and Byrd-Jacobs (2011).

The potential effects of deafferentation on cell fate were examined with quantification of BrdU-positive profiles in the olfactory bulbs 3 weeks after BrdU administration (Fig. 2A). Cell fate comprises differentiation, migration, and/or survival of newly formed cells. In untreated control fish that were allowed to survive for 3 weeks following BrdU

administration, BrdU-positive profiles were present in all layers of the bulb but depicted here in the glomerular and internal cellular layer of the olfactory bulb (Fig. 2B). Anti-KLH was used to label olfactory sensory neuron axons and aid in identification of bulb layers. In fish receiving chronic detergent treatment, a noticeable increase in BrdU-positive profiles in all layers of the olfactory bulbs was found (Fig. 2C). BrdU-positive profiles were abundant in all layers of the deafferented and internal control olfactory bulbs.

Quantitative analysis of the differentiation, migration, and/or survival of newly formed cells in the olfactory bulbs under acute and chronic deafferentation was conducted 3 weeks following the initial Triton X-100 application and concurrent BrdU administration, using BrdU immunoreactivity, the neuronal marker anti-Hu, and the apoptotic cell death marker anti-caspase-3. Counts of BrdU-positive profiles revealed that few newly formed cells were present in the olfactory bulbs of control and acutely deafferented fish, with no significant difference between right and left bulbs in either group (Fig. 2D). However, there was a significant bilateral increase in BrdU-positive profiles in the deafferented and internal control bulbs of chronically deafferented fish. Both olfactory bulbs of these fish were significantly different from those of control and acutely deafferented fish (Fig. 2D). The distribution of newly formed cells differentiating into neuronal and non-neuronal cells was obtained by counting BrdU+ cells that co-labeled with the neuronal marker anti-Hu (Fig. 2E). In untreated control fish, BrdU+/Hu+ double-labeled profiles comprised $57.2\% \pm 1.6$ (left bulb) and $57.8\% \pm 4.1$ (right bulb) of all newly formed cells, with no significant difference observed between sides (Fig. 2F). In acutely deafferented fish, $79.6\% \pm 15.2$ (internal control bulb) and $81.7\% \pm 9.3$ (deafferented bulb) of the total BrdU profiles were co-labeled with anti-Hu, displaying a trend towards a bilateral increase from the co-labeled profiles seen in untreated control fish, although this apparent shift was not significant (Fig. 2F). Chronic deafferentation resulted in a similar trend towards a bilateral increase in BrdU+/Hu+ co-labeled profiles, with $81.8\% \pm 11.2$ (internal control bulb) and $83.0\% \pm 9.1$ (deafferented bulb) of the total BrdU profiles being co-labeled compared to untreated control fish, although no significant difference was seen in any group. Statistical significance was not achieved, likely due to the increased variance seen in the detergent-treated group: compare SEM of 1.6 and 4.1 for control fish and 15.2, 9.3, 11.2, and 9.1 for deafferented fish. A corresponding bilateral reduction in BrdU-positive profiles that did not display a neuronal identity was seen in both deafferented groups, but the shift was not significantly different from the control distribution (Fig. 2F).

Further examination of the location of newly formed neurons within the olfactory bulb layers revealed a shift in distribution of adult-born cells (Fig. 2G). In untreated control fish, BrdU+/Hu+ co-labeled profiles were distributed primarily in the GL, with $83.3\% \pm 9.6$ in the left bulb and $75.8\% \pm 5.5$ in the right bulb, with the remainder of the newly formed neurons found in the ICL. No co-labeled profiles were found in the ONL of these control fish. Following acute deafferentation, the ipsilateral olfactory bulb showed a significant reduction relative to control fish in BrdU+/Hu+ co-labeled profiles in the GL ($41.2\% \pm 7.9$), with a corresponding significant increase in the ICL ($58.8\% \pm 7.9$). The internal control bulb of acutely deafferented fish appeared to exhibit slightly diminished BrdU+/Hu+ co-labeled profiles in the GL ($62.5\% \pm 7.2$) and increased numbers in the ICL ($37.5\% \pm 7.9$) relative to untreated control fish, although not to a level of significance. In chronically deafferented

fish, a small percentage of BrdU+/Hu+ co-labeled profiles ($3.7\% \pm 2$) were observed in the ONL of the internal control bulb. A significant bilateral reduction in BrdU+/Hu+ co-labeled profiles was observed in the GL, with $40.0\% \pm 1.2$ in the internal control bulb and $34.6\% \pm 4.9$ in the deafferented bulb and a corresponding bilateral increase in BrdU+/Hu+ co-labeled profiles in the ICL, with $56.3\% \pm 0.8$ in the internal control bulb and $65.4\% \pm 4.9$ in the deafferented bulb (Fig. 2G).

Additional analysis of cell survival and survival of newly formed cells during chronic deafferentation was performed using the apoptotic cell death marker, anti-caspase-3 in fish that received a BrdU injection concurrent with the first detergent application and were examined 3 weeks later. All dying cells were identified with anti-caspase-3 and newly formed cells undergoing cell death were co-labeled with anti-BrdU (Fig. 3A). In untreated control fish, few caspase-3-positive profiles were found in both of the olfactory bulbs, while a bilateral increase in cell death was found following chronic deafferentation, with significantly more caspase-3-positive profiles found in the deafferented bulb (Fig. 3B). The death of newly formed cells was obtained by counting BrdU-positive cells that co-labeled with anti-caspase-3. A paucity of caspase-3+/BrdU+ co-labeled profiles were found in both of the bulbs of untreated control fish (Fig. 3C). In chronically deafferented fish, a bilateral increase in caspase-3+/BrdU+ co-labeled profiles was seen (Fig. 3C). Further, caspase-3+/BrdU+ co-labeled profiles were significantly increased in the deafferented bulb relative to the internal control bulb. It appears that most of the cell death in untreated control and chronically deafferented fish was of newly formed cells. In control fish, caspase-3+/BrdU+ co-labeled profiles made up about $38.9\% \pm 19.5$ of total caspase-3-positive cells. Following chronic deafferentation, the percent of total caspase-3+ profiles that were caspase-3+/BrdU+ co-labeled profiles, and therefore were newly formed cell that were dying, was $93.8\% \pm 3.6$. No significant difference was found, although it was interesting to note that in the treated side of the chronically deafferented fish all cell death was of newly formed cells.

The potential effects of deafferentation were further examined by evaluating the location within the olfactory bulb layers of dying newly formed cells. In this group of untreated control fish, caspase-3+/BrdU+ labeled profiles were not found in the ONL (Fig. 3D), only a few caspase-3+/BrdU+ labeled profiles were found in the ONL (Fig. 3E), and there were no caspase-3+/BrdU+ labeled profiles found in the ICL (Fig. 3F). In chronically deafferented fish, a significant increase in caspase-3+/BrdU+ co-labeled profiles was seen in the GL of the deafferented olfactory bulb relative to the internal control bulb and bulbs of untreated control fish (Fig. 3E). Caspase-3+/BrdU+ profiles appeared elevated in the GL of the internal control bulb of chronically deafferented fish but were not significantly different from counts from untreated control fish (Fig. 3E). While some caspase-3+/BrdU+ profiles were found in the ONL and ICL of chronically deafferented fish, the counts were not significantly different from control fish (Fig. 3D, F). Collectively, these data indicated that deafferentation affects some aspects of cell fate, namely cell migration and survival.

Next, we took advantage of the reversible nature of the deafferentation elicited with detergent treatment and examined the potential effects of reafferentation following chronic deafferentation. Cell proliferation and fate in the olfactory bulb was investigated using anti-BrdU, anti-Hu, and anti-caspase-3 immunoreactivity. Quantitative analysis of cell

proliferation following reafferentation was conducted in fish that had 3 weeks of detergent treatment, 3 weeks cessation of detergent treatment with BrdU delivery on the last day, and a 4h survival period (Fig. 4A). Control fish received a BrdU injection and had a 4h survival period. Recovery fish displayed few BrdU-positive profiles in the olfactory bulbs (Fig. 4B), with no significant difference from control fish, suggesting little effect of reafferentation on cell proliferation. Our lab previously showed that following 3 weeks of reafferentation olfactory axon innervation and glomerular structure has returned to the control condition (Paskin and Byrd-Jacobs, 2012). In order to determine if the bulb is reinnervated prior to this time point, we performed optical density analysis of anti-KLH labeling 1 week following cessation of detergent treatment (Fig. 4C). The intensity of axonal labeling was not significantly different from control fish, indicating reafferentation of the bulb by 1 week.

The fate of newly formed cells under conditions of reafferentation was assessed in fish that had received 3 weeks of detergent treatment, with BrdU delivery concurrent with the final detergent treatment, and 3 weeks cessation of detergent treatment (Fig. 5A). Control fish received a BrdU injection on the same day as the recovery fish group and had a 3 week survival period. Comparisons were also made to the chronic deafferentation group from Fig. 2. In recovery fish, a significant increase in BrdU-positive profiles was found in both the reafferented bulb and internal control bulb relative to bulbs of untreated control fish (Fig. 5B). Interestingly, no significant difference was found between the bilateral increase in BrdU-positive profiles seen in recovery fish and that observed in chronically deafferented fish (Fig. 2E), suggesting that deafferentation caused the effect on cell fate and reafferentation maintains the effect.

Neuronal differentiation patterns of cells born under conditions of reafferentation were assessed by quantifying newly formed cells that displayed the neuronal marker anti-Hu. As described above, BrdU+/Hu+ co-labeled profiles comprised approximately 60% of all newly formed cells in control fish and 82% in chronically deafferented fish (Fig. 2F). Recovery of afferent input caused a significant bilateral reduction in BrdU+/Hu+ co-labeled profiles in relation to both untreated control and chronically deafferented fish, with $30.4\% \pm 6.5$ in the internal control bulb and $40.5\% \pm 0.47$ in the reafferented bulb of treated fish and $57.2\% \pm 1.6$ in the left bulb and $57.8\% \pm 4.1$ in the right bulb of untreated control fish (Fig. 5C).

Further investigation into the location of newly formed neurons during reafferentation revealed a shift in distribution of these cells in the layers of the olfactory bulb. As described above, chronic deafferentation caused a shift in the location of newly formed neurons, with an increase in the ICL and a reduction in the GL (Fig. 2G). Recovery of afferent input revealed a return to near control distribution (Fig. 5D), with no significant between control and recovery groups. There was, however, a significant reduction in BrdU+/Hu+ co-labeled profiles in the ICL and a significant increase in BrdU+/Hu+ profiles in the GL relative to chronic deafferentation fish (Fig. 2G).

The survival of newly formed cells under conditions of reafferentation was investigated with quantitative analysis of caspase-3-positive profiles that co-labeled with anti-BrdU. As reported above, very few dying newly formed cells were seen in both olfactory bulbs of control fish, while chronic deafferentation caused a significant bilateral increase in the death

of newly formed cells (Fig. 3C). In recovery fish, no caspase-3+/BrdU+ co-labeled profiles were seen. While 0 co-labeled profiles in recovery fish was not significantly different from 1.7 ± 1.2 (left bulb) and 0.67 ± 0.33 (right bulb) co-labeled profiles in untreated control fish, there was a significant bilateral reduction in number of caspase-3+/BrdU+ profiles in recovery fish relative to chronically deafferented fish. Altogether, these data suggest that reafferentation affected cell fate.

While performing preliminary studies, we observed that fish taken from another population, which was older than those used in the current study, showed patterns of cell genesis that were not consistent with the younger fish used for the rest of the study. We then did an additional analysis of BrdU, Hu, and caspase-3 immunoreactivity of older fish (12–15 months) and younger fish (5–9 months) to determine whether age of the fish affects the fate of newly formed cells. Three weeks following BrdU delivery, the number of BrdU-positive profiles found in both of the olfactory bulbs of older fish was commensurate with that found in younger fish (Fig. 6A). To investigate whether age of the fish had an effect on the identity of newly formed cells, BrdU-positive profiles expressing the neuronal marker anti-Hu were examined. In the olfactory bulbs of younger fish, the BrdU+/Hu+ profile distribution was $57.2\% \pm 1.6$ in the left bulb and $57.9\% \pm 4.1$ in the right bulb (Fig. 6B), showing a nearly even distribution in newly formed cells that became neurons and non-neuronal cells. However, in older fish, a shift in differentiation was seen, with a significant increase in newly formed neurons, with $90.1\% \pm 1.6$ in the left bulb and $93.3\% \pm 3.4$ in the right bulb (Fig. 6B). A corresponding significant decrease in newly formed cells was seen in both olfactory bulbs, with an approximately 34% decrease in BrdU-positive cells relative to younger fish.

Analysis of the location of newly formed neurons throughout the olfactory bulb layers of younger and older fish revealed a trend toward a shift in distribution, although not to significance (Fig. 6C). In younger fish, BrdU+/Hu+ profiles were found in the GL and ICL. BrdU+/Hu+ profiles were not found in the ONL. The preponderance of BrdU+/Hu+ profiles were found in the GL, with $83.3\% \pm 9.6$ in the left bulb and $75.8\% \pm 5.5$ in the right bulb. The ICL showed a small percentage of BrdU+/Hu+ profiles, with $16.7\% \pm 9.6$ in the left bulb and $24.2\% \pm 5.5$ in the right bulb. Relative to younger fish, the distribution of BrdU+/Hu+ profiles in older fish showed a trend toward reduced numbers in the GL and increased numbers in the ICL. Few BrdU+/Hu+ profiles were found in the ONL of older fish, with $14\% \pm 9$ in the left bulb and $6.7\% \pm 6.7$ in the right bulb. The majority of BrdU+/Hu+ profiles were seen in the GL, with $61.8\% \pm 2.8$ in the left bulb and $51.7\% \pm 10.8$ in the right bulb. A moderate level of BrdU+/Hu+ profiles were present in the ICL, with $24.3\% \pm 10.2$ in the left bulb and $41.7\% \pm 4.1$ in the right bulb (Fig. 6C).

Whether fish age was a possible influence on cell survival was investigated by quantitative analysis of caspase-3 immunoreactivity. In younger fish, few caspase-3 profiles were found in the olfactory bulbs (Fig. 6D). While caspase-3-positive profiles appeared diminished in older fish, it was not to a level of significance (Fig. 6D). To further elucidate any potential age-related influence on cell survival, we investigated cell survival in both younger and older fish under conditions of chronic deafferentation. The potential effect of age on cell survival in chronically deafferented fish was examined by quantitative analysis of cells displaying

anti-caspase-3. In younger fish, chronic deafferentation caused a moderate level of caspase-3-positive profiles in the left and right olfactory bulbs (Fig. 6D). In older fish, there were significantly fewer caspase-3-positive profiles in both olfactory bulbs following chronic deafferentation than was seen in older fish (Fig. 6D). Taken together, these data suggest that age of fish affected the fate of newly formed cells in both control and chronic deafferentation conditions.

DISCUSSION

The present study investigated the process of cell genesis as a contributing factor in the deafferentation-induced reduction in olfactory bulb volume and reafferentation-induced restoration of bulb volume in adult zebrafish (Paskin et al., 2011). We found that migration and survival of newly formed cells in the olfactory bulb are altered by persistent long-term deafferentation, whereas reafferentation alters patterns of newly formed cell migration and differentiation. Further, fish age is shown to be a contributing factor in differentiation and survival of newly formed cells.

Our data reveal that following acute and chronic deafferentation and reafferentation, cell proliferation within the olfactory bulb is not significantly different from control levels. This result is similar to what has been previously reported regarding cell proliferation levels found in the olfactory bulbs 4h following BrdU administration at short-term (4h) and long-term (3 week) survival times after permanent, complete deafferentation (Villanueva and Byrd-Jacobs, 2009). The proliferating cells within the adult zebrafish olfactory bulbs do not contribute significantly to the deafferentation-induced reduction in bulb volume or to the reafferentation-induced recovery in bulb volume. The olfactory bulb is only 1 of 16 identified proliferation niches in adult zebrafish, making it possible that an increase in proliferation and/or migration may be occurring in another proliferation niche (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006).

Chronic deafferentation, with repeated detergent irrigation of the olfactory organ, causes an increase in newly formed cells in both olfactory bulbs. Reafferentation, following cessation of detergent treatment, appears to sustain or initiate the chronic deafferentation-induced bilateral increase in migration of newly formed cells into the olfactory bulbs. Comparable to the mammalian system, neuronal precursor cells in adult zebrafish migrate from the telencephalic ventricular zone through the rostral migratory stream and into the olfactory bulbs (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006; Kishimoto et al., 2011). In conjunction with the low cell proliferation levels we reported within the olfactory bulb, the increase in newly formed cells found in the olfactory bulbs following 3 weeks of chronic deafferentation and following 3 weeks of recovery suggests that these cells originate in the telencephalic ventricular zone and then migrate into the bulb. In juvenile rats, the return of afferent input following sensory deprivation shows a similar increase in newly formed cells but only in the recovering sensory-deprived bulb (Cummings et al., 1997). Not only did we find an increase in newly formed cells in the olfactory bulb ipsilateral to the lesion, but we also found an unexpected chronic deafferentation- and reafferentation-induced increase in newly formed cells in the contralateral bulb. Effects of experimental manipulations contralateral to damage are not unusual. In adult mice, unilateral bullectomy results in a

transient increase in proliferation rate in the olfactory epithelium on the contralateral side (Carr and Farbman, 1992). A bilateral increase in newly formed cells is found in the olfactory bulb portion of the rostral migratory stream following unilateral axotomy (Mandairon et al., 2003). The multi-synaptic connections that exist between the olfactory bulbs through the anterior olfactory nucleus and anterior commissure in mice may account for this apparent crosstalk (Haberly and Price, 1978). In zebrafish, mitral cells project their axons to multiple targets in the forebrain including the subpallium of the ventral telencephalon and across the midline through the anterior commissure resulting in branches in both hemispheres of the telencephalon (Rink and Wullmann, 2004; Miyasaka et al., 2009). Thus, altered activity in the chronically deafferented olfactory bulb (Paskin et al., 2011) may modify the synaptic input to the contralateral side of the telencephalon, potentially adjusting the ventricular zone activity that subsequently influences the bulb. The potential alterations in activity in the contralateral olfactory bulb could account for the bilateral increase in migration of newly formed cells into the bulb. In support of this, another form of deafferentation of the zebrafish olfactory bulb also suggested a bilateral increase in migration (Villanueva and Byrd-Jacobs, 2009).

Acute deafferentation with a single dose of Triton X-100 does not elicit the dramatic effects of chronic deafferentation caused by repeated exposure to the detergent. Newly formed cells are not increased in the olfactory bulbs 3 weeks following acute deafferentation. This is likely due, in part, to the rapid restoration of afferent input to the olfactory bulb with this lesion. Following a single treatment with Triton X-100, the olfactory epithelium is regenerated within 5 days (Iqbal and Byrd-Jacobs, 2010) and reinnervation of the olfactory bulb within 7 days (White et al., 2015). If acute deafferentation produces cell death in the deafferented olfactory bulb, precursor cells that have divided prior to the injury and are already migrating to the bulb may participate in the regenerative process, as shown in adult teleost cerebellum (Zupanc and Ott, 1999).

Deafferentation appears to enhance the percentage of newly formed cells expressing a neuronal fate, while reafferentation causes a reduction in newly formed cells expressing a neuronal fate, in both olfactory bulbs. We also found that deafferentation alters the distribution of newly formed neurons in the layers of the olfactory bulbs, while reafferentation reveals a return to the distribution found in control fish. More newly formed cells adopting a neuronal identity are found in the ICL than in the GL in the ipsilateral bulb of acutely deafferented fish and in both olfactory bulbs following chronic deafferentation. This may be due to alterations in differentiation of cells into interneurons or survival of specific neuronal types influenced by the remaining connections. In adult mice, sensory deafferentation by naris occlusion does not affect neuronal distribution in the olfactory bulb or the distribution of newly formed neurons in the bulb layers (Mandairon et al., 2006). This discrepancy may be the result of differing time points since our study observed effects at 21d after BrdU exposure, while the mouse study examined neuronal fate and distribution after 45d and further modification of cell fate may have occurred. Another reason for differences in our findings may be due to type of afferent removal: our detergent lesions remove most afferent axons while naris occlusion reduces activity without removing afferent axons. Commitment to neuronal fate appears to be influenced directly or indirectly by the significant alteration that chronic deafferentation followed by reafferentation exerts on the

morphology and physiology of the olfactory bulb in our model (Paskin et al., 2011; Paskin and Byrd-Jacobs, 2012).

We found that the death of newly formed cells increases in both olfactory bulbs following chronic deafferentation, with more dying new cells in the deafferented bulb (especially notable in the GL) compared to the internal control bulb. It is well established that the survival of newly formed cells in adult mice is dependent upon sensory input and formation of synaptic connections, although specific mechanisms have yet to be elucidated (Corotto et al., 1994; Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Mandaïron et al., 2003; 2006). In adult mice, a similar reduction in newly formed cells in the GL is observed following naris occlusion (Mandaïron et al., 2006), whereas sensory deprivation achieved by genetically disrupting olfactory transduction (Petreanu and Alvarez-Buylla, 2002) shows an increase in the death of newly formed cells in the granule cell layer. In adult zebrafish, chronic deafferentation causes a reduction in innervation by olfactory sensory neuron axons (Paskin and Byrd-Jacobs, 2012) resulting in a reduction of neuronal activity (Paskin et al., 2011). In the deafferented bulb, the only cell death observed is that of newly formed cells. Repeated intranasal irrigation with detergent results in reduced bulb volume, the number of glomeruli, and tyrosine hydroxylase expression, an indicator of afferent activity (Paskin et al., 2011; Paskin and Byrd-Jacobs, 2012). These alterations to the microenvironment of the bulb may reduce the ability for newly formed cells to establish or maintain a connection in the reduced neuronal circuitry resulting in cell death. We did not find evidence of death of existing cells in these bulbs. In zebrafish, the olfactory epithelium thickness and antibody labeling for neurons is reduced one day following a single intranasal irrigation with detergent (Iqbal and Byrd-Jacobs, 2010) resulting in an immediate reduction in afferent input and subsequent denervation of the bulb. Thus, during three weeks of chronic deafferentation the majority of death of existing cells has most likely already occurred. Since we found an increase in the number of newly formed cells in the intact internal control bulb, the increased death of these newly formed cells may be part of the innate culling of super-numerous newly formed cells that are not needed in the intact bulb. In adult rats, under physiologic conditions approximately 50% of newly formed neurons are eliminated upon reaching the olfactory bulb (Biebl et al., 2000; Winner et al., 2002).

While chronic deafferentation decreased cell survival, we did not find any evidence of death of newly formed cells upon reafferentation, although this finding is not significantly different from control levels. It is well established that sensory input promotes survival of neurons during development in vivo (Gould et al., 1994; Najbauer and Leon, 1995; Ikonomidou et al., 1999; Adams et al., 2004) and in vitro (Balazs et al., 1988), but examples in adults are less common. Adult mice exposed to odor-enriched environments show an increase in newly formed cell survival (Rochefort et al., 2002). Similarly, new neuronal survival is regulated in an input-dependent manner in the dentate gyrus of adult mice that may enable them to play a role in learning and memory (Tashiro et al., 2006). Thus, reafferentation may alter the bulb microenvironment such that the survival of newly formed neurons is increased and these cells contribute, in part, to the recovery of olfactory bulb volume following chronic deafferentation.

In total, these data suggest that chronic deafferentation causes a bilateral increase in the migration of newly formed cells, most of which become neurons, into the olfactory bulbs and that the vast majority of these cells undergo cell death. Reafferentation maintains or initiates a bilateral increase in migration of newly formed cells into the olfactory bulbs, with the vast majority of these cells being of non-neuronal identity and not dying. The addition of these newly formed cells likely contributes, in part, to the recovery of olfactory bulb volume following recovery from chronic deafferentation. Further, the bilateral increase found following both chronic deafferentation and reafferentation indicates that the adult zebrafish may employ the overproduction of newly formed cells and cell death paradigm that is similarly found during development (Oppenheim, 1991; Jacobson et al., 1997).

The impact of aging in the zebrafish brain is not extensively studied, so fish age as a potential factor in cell genesis and death was examined. We found that the age of the fish does not affect the number of newly formed cells in the olfactory bulbs but does affect their identity. In mice, age induces a reduction in formation of newly formed cells in the subventricular zone and, subsequently, in the olfactory bulbs in 2- to 4-month-old vs. 20- to 24-month-old mice (Tropepe et al., 1997; Jin et al., 2003). Developmental and physical maturity in mice is defined as complete at 3- to 6-months of age (Harrison: "Maximum Lifespan as a Biomarker of Aging"; The Jackson Laboratory, 2011), with an average lifespan of 2 years. In 2- to 4-month old mice, some maturation may still be occurring but is clearly complete at 20- to 24-months of age. In zebrafish, growth continues throughout life with an average 2-year lifespan, with the growth rate being greatest at 0- to 3-months of age and then beginning to decline, approaching zero at around 18 months of age (Spence et al., 2007). Thus, the continued growth of adult zebrafish may minimize or delay any potential age-related decline in cell genesis in the olfactory bulbs at the time points we examined. The older fish we examined were 12- to 15-months old. An examination of 18-month or older fish may better reveal additional potential age-related influence on cell migration, although those studies are challenging due to the increased mortality of older fish following extensive experimental manipulations such as those in this study.

Interestingly, age does influence the differentiation and survival of newly formed cells. We found that older fish produce more newly formed cells displaying a neuronal identity. Another study on the effect of aging on neurogenesis in zebrafish reported that the number of newborn neurons in the olfactory bulb remains constant at 6–10 months of age and declines at 20 months of age (Edelmann et al., 2013). The difference in findings from our result may be due to experimental differences in age of fish examined and in that we quantified BrdU-ir profiles 3 weeks following BrdU administration, whereas that study quantified newly formed neurons 6 days following BrdU administration. We found no effect of fish age on cell survival in the intact olfactory bulb, but we saw increased cell death in younger fish compared to older fish under chronic deafferentation conditions. In mammals, the transplantation of neurons from mouse brain into rat brain demonstrates that the lifespan of neurons is limited by the maximal lifespan of the longer-living host organism and not the maximal lifespan of the donor organism (Magrassi et al., 2013). Therefore, the aging microenvironment of the specific organism affects neuronal survival. Taken together, aging does not affect the number of migrating cells into olfactory bulb but does alter their ability to survive and influences their identity.

In light of the potential ongoing growth-induced minimization or delay of aging effects, we looked at the potential effects of aging on cell survival under conditions of chronic deafferentation. Chronic deafferentation causes more cell death in younger fish than in older fish. Therefore, alterations in the olfactory bulb resulting from chronic deafferentation may amplify any potential age-related hastening of cell death and, at this time point, the majority of cell death in the older bulb may have already occurred. Taken together, fish age does not affect the number of cells migrating into the olfactory bulb but does alter their ability to survive and their identity.

In sum, our results show that, contrary to our hypothesis, chronic deafferentation increased the migration of newly formed cells into the olfactory bulb, but a number of these cells did not survive. Fish age affected the identity of newly formed cells and their ability to survive. Reafferentation either sustained or initiated a reafferentation-induced increase in migration of newly formed cells into the olfactory bulb and enhanced their ability to survive, supporting our hypothesis. Thus, newly formed cells contribute, in part, to the recovery of olfactory bulb volume following chronic deafferentation.

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Abbreviations

BrdU	5-bromo-2'-deoxyuridine
GL	Glomerular Layer
ICL	Internal Cellular Layer
KLH	Keyhole limpet hemocyanin
PBS	Phosphate-Buffered Saline
ONL	Olfactory Nerve Layer

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Figure 1.

Analysis of cell proliferation in the olfactory bulb during acute and chronic deafferentation. (A) The untreated control group received BrdU at time 0 and was examined 4h later. The acute deafferentation treatment group received concurrent BrdU and Triton X-100 treatment at time 0 and was examined 4h later. The chronic deafferentation treatment group received Triton X-100 treatments every 3 days for 3 weeks and BrdU administration concurrent with the final treatment, followed by a 4h survival period. (B) Cell proliferation in the olfactory bulb was not altered by acute or chronic deafferentation. Low levels of newly formed cells

were found in both olfactory bulbs in all groups and no significant differences were found. (C) Olfactory sensory neuron axons were visualized with anti-KLH in coronal sections of the bulb; dorsal is to the top and ventral is to the bottom. In the chronically deafferented bulb, innervation was greatly diminished on the medial side of the bulb (arrows) and substantially diminished on the lateral side of the bulb (arrowheads) relative to the internal control bulb. Scale bar=50µm; TX=Triton X-100.

Figure 2. The effects of acute and chronic deafferentation on cell fate in the olfactory bulb. (A) The untreated control group received BrdU on day 0 and was examined after 3 weeks. The acute deafferentation group was administered BrdU on day 0 concurrently with detergent treatment and was examined after 3 weeks. The chronic deafferentation group received BrdU and the initial detergent treatment concurrently on day 0, followed by detergent treatment every 3 days for 3 weeks before being examined. (B) The zebrafish olfactory bulb is comprised of 3 diffusely organized concentric circles, the olfactory nerve (ONL),

glomerular (GL) and internal cellular (ICL) layers. In semi-serial sections of a control fish examined 3 weeks after BrdU administration, few newly formed cells (arrows) were seen throughout the olfactory bulb. Olfactory sensory neuron axons were visualized with anti-KLH (brown). A higher magnification of the indicated region is shown in B'. (C) In semi-serial sections of a chronically deafferented fish, abundant BrdU-positive profiles (arrows) were observed in all layers of the olfactory bulb. Olfactory sensory neuron axons were labeled with anti-KLH (brown). A higher magnification of the indicated region is shown in C'. (D) In control and acutely deafferented fish, quantitative analysis revealed that few newly formed cells were present in either of the olfactory bulbs at 3 weeks after BrdU administration. Chronic deafferentation resulted in a significant bilateral increase in newly formed cells at this time point. (E) Anti-BrdU (green) and anti-Hu (magenta) identified newly formed neurons with confocal microscopy. (F) In control fish, approximately 60% of newly formed cells examined 3 weeks after birth had become neurons. While the neuronal identity of newly formed cells appeared elevated following both detergent treatments, with approximately 80% displaying a neuronal marker, there was no significant difference from control fish. (G) The distribution of newly formed neurons in the GL and ICL of the olfactory bulbs was altered by deafferentation. In control fish approximately 80% of newly formed neurons were found in the GL and 20% in the ICL, and no new neurons were found in the ONL. Acute deafferentation caused a significant shift in neuronal distribution where newly formed neurons significantly increased in the ICL to approximately 60% and decreased in the GL to approximately 40% on the side ipsilateral to the detergent treatment only; newly formed neurons appeared to show a similar shift in distribution in the ICL and GL in the internal control olfactory bulb, although counts were not significantly different from control fish or the deafferented bulb. Three weeks of detergent treatment resulted in a bilateral shift in distribution of newly formed neurons, with a significant increase in the ICL to approximately 60% and a significant decrease in the GL to approximately 37%. Scale Bar=50µm (B,C,E) or 10µm (B',C'); $P<0.05$; a = significantly different from control; b= significantly different from single TX.

Figure 3.

Cell survival is altered during chronic deafferentation. (A) Anti-BrdU (green) and anti-caspase-3 (red) identified dying cells and dying newly formed cells with confocal microscopy. In an untreated control bulb, a BrdU-positive profile is also caspase-3 labeled (arrow). (B) In a chronically deafferented fish, there are numerous BrdU-positive profiles and several caspase-3-positive profiles; only some profiles overlap (arrow). (C) In control fish, dying cells were sparse in both olfactory bulbs. Chronic deafferentation resulted in a significant bilateral increase in the number of cells expressing a cell death marker. Further,

the deafferented bulb had significantly more dying cells than the internal control bulb. (D) Few newly formed cells that expressed a cell death marker were found in both bulbs of control fish, while a bilateral increase in the death of newly formed cells was found in both bulbs of chronically deafferented fish. Additionally, more dying newly formed cells were found in deafferented bulb relative to the internal control bulb. (E) Dying newly formed cells were not found in the ONL of either olfactory bulb in control fish and were found only at very low levels in chronically deafferented fish. (F) In the GL of control fish, few newly formed cells displayed a cell death marker, but in chronically deafferented fish, significantly more death of newly formed cells was found in the GL of the deafferented bulb relative to the internal control bulb and control fish. (G) Dying newly formed cells were not observed in the ICL of either olfactory bulb in control fish, and in chronic deafferentation fish only low levels of dying newly formed cells were observed in the ICL of both olfactory bulbs. Scale bar=20 μ m. $P<0.05$; * = within group significance; a = significantly different from control.

Figure 4.

Analysis of cell proliferation following reafferentation. (A) The untreated control group received BrdU at time 0 and was examined 4h later. The recovery group had detergent treatments beginning on day 0 and continuing every 3 days for 3 weeks. The detergent treatments then ceased for 3 weeks to permit recovery of innervation. BrdU was administered at 6 weeks and examined 4h later. (B) In the recovery fish, low levels of newly formed cells were seen in the reafferented and internal control bulbs, with no significant difference from control fish. (C) By 1 week of recovery following 3 weeks of detergent

treatment, olfactory sensory neuron innervation of the olfactory bulb was near control levels, as measured by anti-KLH intensity.

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Figure 5.

Restoration of afferent input affects cell fate. (A) The untreated control group received BrdU on day 0 and was examined after 3 weeks. The recovery group had detergent treatments beginning at day 0 and continuing every 3 days for 3 weeks, with BrdU administration concurrent with the final treatment. The detergent treatments ceased for 3 weeks before examination. (B) Recovery of afferent input resulted in a significant bilateral increase in the number of newly formed cells in the olfactory bulbs relative to control fish. This effect was significant from control fish but was not significantly different from the increase seen following chronic deafferentation (Fig. 3). (C) In recovery fish, a significant bilateral reduction in newly formed cells with a neuronal identity, and a corresponding increase in newly formed cells with a non-neuronal identity, was seen relative to control fish. (D) The distribution of new neurons in the olfactory bulb layers in recovery fish was not significantly different from the distribution found in control fish. $P < 0.05$; a = significantly different from control.

Figure 6.

Age of fish impacts cell fate. (A) Nearly equal numbers of newly formed cells were found in both sides of the olfactory bulbs of the younger (5–9 months) and older (12–15 months) fish. (B) Older fish produced significantly more newly formed neurons than younger fish. (C) Neuronal distribution in older control fish appeared to be diminished in the GL and elevated in the ICL relative to younger fish, although not to a significant extent. (D) Younger chronically deafferented fish displayed a significant bilateral increase in cell death. $P < 0.05$; a = significantly different from older fish; b = significantly different from younger chronically deafferented fish.