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Peripheral Axons of the Adult Zebrafish Maxillary Barbel Extensively Remyelinate During Sensory Appendage Regeneration

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Abstract

Myelination is a cellular adaptation allowing rapid conduction along axons. We have investigated peripheral axons of the zebrafish maxillary barbel (ZMB), an optically clear sensory appendage. Each barbel carries taste buds, solitary chemosensory cells, and epithelial nerve endings, all of which regenerate after amputation (LeClair and Topczewski [2010] PLoS One 5:e8737). The ZMB contains axons from the facial nerve; however, myelination within the barbel itself has not been established. Transcripts of myelin basic protein (*mbp*) are expressed in normal and regenerating adult barbels, indicating activity in both maintenance and repair. Myelin was confirmed in situ by using toluidine blue, an anti-MBP antibody, and transmission electron microscopy (TEM). The adult ZMB contains ~180 small-diameter axons (<2 μ m), approximately 60% of which are myelinated. Developmental myelination was observed via whole-mount immunohistochemistry 4–6 weeks postfertilization, showing myelin sheaths lagging behind growing axons. Early-regenerating axons (10 days postsurgery), having no or few myelin layers, were disorganized within a fibroblast-rich collagenous scar. Twenty-eight days postsurgery, barbel axons had grown out several millimeters and were organized with compact myelin sheaths. Fiber types and axon areas were similar between normal and regenerated tissue; within 4 weeks, regenerating axons restored ~85% of normal myelin thickness. Regenerating barbels express multiple promyelinating transcription factors (*sox10*, *oct6* = *pou3f1*; *krox20a/b* = *egr2a/b*) typical of Schwann cells. These observations extend our understanding of the zebrafish peripheral nervous system within a little-studied sensory appendage. The accessible ZMB provides a novel context for studying axon regeneration, Schwann cell migration, and remyelination in a model vertebrate.

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AUTHOR ROLES AND RESPONSIBILITIES

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Additional Supporting Information may be found in the online version of this article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Keywords

Schwann cell; taste bud; *sox10*, *pou3f1*, *egr2a/b*

Myelination is a cellular adaptation of vertebrate nervous systems allowing rapid electrical conduction along axons. Myelination occurs in both the central nervous system (CNS) and the peripheral nervous system (PNS) when glial cells—oligodendrocytes and Schwann cells, respectively—wrap thin cytoplasmic processes around developing axons. Although oligodendrocytes and Schwann cells establish myelin during embryogenesis and maintain it during maturity, these cells are also studied for their ability to inhibit or enhance myelin repair. In mammals, oligodendrocytes inhibit CNS remyelination (Chen et al., 2002), whereas Schwann cells promote PNS remyelination (Gordon and Gordon, 2010; Hall, 1989). Strategies to enhance myelination include promoting the endogenous repair mechanisms of Schwann cells in the PNS, or introducing Schwann cells into the injured CNS (for review see Stangel and Hartung, 2002). Understanding the repertoire of Schwann cell behavior in multiple contexts is thus important for both CNS and PNS myelination.

Although much has been learned about the molecular basis of remyelination, many regulatory processes remain to be studied. The zebrafish is a popular species in which to investigate these processes, many of which are evolutionarily conserved (Avila et al., 2007; Buckley et al., 2008; Dubois-Dalcq et al., 2008). Zebrafish axons are myelinated by the same glial cell types as in mammals (Buckley et al., 2008; Jeserich et al., 2008), and the embryonic origins and migrations of these cell types are understood in detail (Woodhoo and Sommer, 2008). The spatial and temporal expression of myelin in early embryos and larvae has been mapped (Brösamle and Halpern, 2002; Buckley et al., 2010), and the molecular similarities and differences between zebrafish and mammalian myelin are increasingly well characterized (Avila et al., 2007; Bai et al., 2011; Buckley et al., 2008; Jeserich et al., 2008; Schaefer and Brösamle, 2009). Although there are species-specific differences in myelin composition, the function of myelin is highly conserved, as is the network of transcription factors regulating glial cell differentiation and myelin production (Levavasseur et al., 1998; Li et al., 2007). Recently, zebrafish mutagenesis screens have identified specific genes required for myelinating the CNS and/or PNS (Kazakova et al., 2006; Larson et al., 2010; Lyons et al., 2005; Monk and Talbot, 2009; Pogoda et al., 2006), for developing oligodendrocytes (Takada and Appel, 2010; Zhao et al., 2010), and for establishing the nodes of Ranvier (Woods et al., 2006). Transgenic lines expressing fluorescent proteins allow zebrafish axons (Goldman et al., 2001), oligodendrocyte lineages (Park et al., 2007; Yoshida and Macklin, 2005), and/or transcripts of myelin basic protein (Jung et al., 2010) to be easily observed in vivo. Finally, the rapid embryonic development of zebrafish facilitates whole-organism assays of behaviors, such as larval escape swimming, that depend on fast conduction of action potentials (Fetcho, 2007).

Most studies of zebrafish myelination address the embryonic stages, up to 4-5 days postfertilization. Less is known, however, about how myelination is regulated in zebrafish adults. An exception is the zebrafish optic nerve, which, in contrast to mammals, both regenerates and remyelinate after injury (for review see Matsukawa et al., 2004; Nona et al., 1992, 2000). Similarly, the adult zebrafish caudal fin regenerates myelinated axons of the lateral line (Dufourcq et al., 2006). These adult systems are useful for comparing how developmental myelination that occurs in the intact embryo is distinct from remyelination occurring in the context of injury, inflammation, and scarring. The identification of genes that are expressed in adult regenerating tissues, but not in the same tissues embryonically, has led to the notion of “injury-induced molecular programs” with distinct regulatory controls (for review see Kizil et al., 2011). This idea is further supported by experiments in

vivo in which embryonically important genes were knocked down, with no effect on adult myelin maintenance or regeneration (Atanasoski et al., 2006; for review see Taveggia et al., 2010). These data suggest that multiple, intersecting molecular pathways regulate myelin at different stages of the life cycle, highlighting the need for further studies of this phenomenon.

We have introduced the zebrafish maxillary barbel (ZMB) as a novel system in which to investigate nerve, skin, and vascular regeneration (LeClair and Topczewski, 2009, 2010). Barbels first develop in zebrafish juveniles 30-40 days postfertilization and remain optically transparent throughout the life cycle. There are no intrinsic muscles and few other cell types in the barbel, making the neural architecture of these appendages exceptionally visible. The larger maxillary barbel is innervated by many small-diameter axons descending from the facial nerve (cranial nerve VII). Most of these axons project ventrally to innervate taste buds; other neural cell types in the barbel include solitary chemosensory cells (SCCs) and a diffuse epithelial nerve net. After a proximal amputation in which more than 85% of the maxillary barbel is removed, the appendage regenerates all of these structures within 2-3 weeks at 28°C (Fig. 1; see also Fig. 10 of LeClair and Topczewski, 2010). Interestingly, the central axons of the barbel grow out several millimeters despite extensive scarring of the appendage and a permanent disorganization of mesodermal cells and associated matrix. This makes the ZMB a convenient structure in which to study how peripheral axons navigate the extracellular environment and establish connections to distal sensory targets.

Having previously documented robust regrowth of peripheral axons into regenerating barbel tissue, we wished to determine whether these axons were originally myelinated and whether, after regeneration, myelination was restored. Our experimental goals were 1) to assess the myelination state of adult, developing, and regenerating ZMB axons; 2) to observe the time course of remyelination during barbel regeneration; 3) to determine whether regenerated axons were similar to normal axons in size, fiber type, and myelin thickness; and 4) to test whether regenerating barbel tissue expresses conserved transcription factors, including *sox10*, *pou3f1* (= *oct6*), and *egr2a/b* (= *krox20a/b*), all characteristic of myelinating peripheral Schwann cells (Kawasaki et al., 2003; Mande-makers et al., 1999; Monk et al., 2009; Monk and Talbot, 2009; Svaren and Meijer, 2008). Although intensively studied in embryos, the expression of these genes in zebrafish adults is not well mapped. Zebrafish *pou3f1*, for example, has curated expression data only up to larval day 5 (www.zfin.org). We therefore sought to determine whether these transcripts were active in regenerating adult barbels, indicating reactivation of the embryonic myelinating regulatory program. If demonstrated, these features would make the ZMB an attractive context in which to study the cellular and molecular basis of remyelination in the adult zebrafish PNS, enhancing the utility of this popular model organism.

MATERIALS AND METHODS

Zebrafish husbandry

Wild-type “AB” zebrafish were bred and reared under standard conditions at 28°C at DePaul University and/or Children’s Memorial Research Center (Chicago, IL). Larval fish were fed live rotifers and/or a commercial powdered diet once daily. Juvenile and adult fish were fed fish flakes and/or brine shrimp once or twice per day. All animal protocols were approved by the Institutional Animal Care and Use Committees.

Surgical procedures and tissue collection

To collect developing barbel tissue, juvenile zebrafish (30-90 days postfertilization) were anesthetized in 0.015% buffered Tricaine (MS-222; Sigma, St. Louis, MO), then killed in

ice water. Under a stereoscope, the head was removed with a small scalpel or razor blade; larger specimens were bisected sagittally before fixation. To collect regenerating barbel tissue, adult zebrafish 3-12 months postfertilization were lightly anesthetized as described above, and then the left maxillary barbel of each fish was removed by proximal amputation as previously described (LeClair and Topczewski, 2009). The contralateral barbel was left intact as a control. Fish were returned to the system after several hours and were kept for 10 days, 28 days, or 70 days after surgery. On the day of collection, the fish were anesthetized as described above, then killed in ice water. Under a stereoscope, regenerating and control barbel tissues were dissected away from the head and processed as needed for RNA isolation or tissue fixation.

RNA isolation from normal and regenerating maxillary barbels

Freshly dissected adult maxillary barbels were rinsed in saline and transferred with forceps into ~200 μ l of RNeasy lysis buffer (Qiagen, Crawfordsville, IN) in a glass depression slide. Within this solution, the barbels were trimmed under a dissecting scope. Normal barbels were trimmed to exclude the maxilla but otherwise contained all of the cell types in the normal adult appendage. Regenerated barbels were trimmed to exclude the proximal stump of non-regenerating tissue, leaving only cells that had grown out distal to the amputation plane. As previously described, this plane is easily seen under transmitted light (LeClair and Topczewski, 2010). After trimming, the desired fragments were collected with fine forceps, blotted on a Kimwipe to remove excess fluid, and transferred to 250 μ l RNeasy lysis buffer at the bottom of a 1.5-ml RNeasy-free Eppendorf tube. The bottom of the tube was then snapfrozen in a bath of liquid nitrogen and stored at -80°C until all tissues had been collected. As a positive control for zebrafish myelin, we also collected whole larval zebrafish at 5 days postfertilization (5 dpf). Approximately 50 anesthetized embryos were pipetted into a 1.5-ml tube, and the supernatant was removed. Two hundred fifty microliters of fresh RNeasy lysis buffer was then added, and the tube was snap-frozen as described above. After all of the tissues had been collected and frozen, each 250- μ l aliquot of RNeasy lysis buffer was individually thawed until it was just liquid, then ground on wet ice with a motorized RNeasy-free micropestle (Kontes Pellet Pestle). The final volume in each tube was adjusted upward to 1 ml either by adding fresh RNeasy lysis buffer or by combining up to four 250- μ l aliquots of similar homogenates. Homogenates were stored at -20°C for up to several weeks, then processed for total RNA isolation according to the manufacturer's protocol. RNA pellets were resuspended in nuclease-free water, checked for concentration and purity on a Nanodrop 2000c, and stored in aliquots at -80°C . On average, 1-2 μ g total RNA could be obtained from the distal tips of ~30 regenerating barbels, compared with 3-4 μ g from the intact contralateral controls from the same animals.

Reverse-transcription polymerase chain reaction

Gene-specific primers were designed to amplify zebrafish *myelin basic protein (mbp)*, *sox10*, *pou3f1* (=oct6), *egr2a* (=krox20a), and *egr2b* (=krox20b). Primers for *mbp* were taken from Buckley et al. (2010). Primers for other genes were designed with NCBI Primer BLAST and/or Primer 3. To avoid false-positive results from genomic contamination, we designed intron-spanning or exon-junction primers when possible. However, because one of our target genes (*pou3f1*) lacked introns, all RNA samples were additionally treated with DNase (DNA-Away kit; Ambion) prior to amplification. A list of all primer combinations, with expected fragment sizes, is given in Table 1.

Primers were ordered from Integrated DNA Technologies (Coralville, IA) and resuspended in Tris-EDTA (pH 7.5) to a stock concentration of 10 μ M. RT-PCR was performed in a single-tube format (One-Step RT-PCR; Invitrogen, Carlsbad, CA) in an Applied Biosystems (Foster City, CA) 2720 Thermal Cycler. Each 10- μ l reaction included 50-100 ng total RNA,

0.2 μ l each of the forward and reverse primers, 5 μ l of the RT-PCR master mix, and nuclease-free water. Amplification followed the kit's recommended cycling parameters, with adjustments for the annealing temperature (T_m) of each primer pair. We used touchdown PCR for the first 20 cycles, decreasing annealing temperatures from $T_m + 8^\circ\text{C}$ to $T_m - 2^\circ\text{C}$. The last 20 cycles were carried out at the final annealing temperature ($T_m - 2^\circ\text{C}$). After amplification, the entire reaction volume was run on a 1.5% agarose gel in lithium-boric acid (LB) buffer (Brody et al., 2004) with ethidium bromide. Images were obtained on an AlphaInnotech FluorChem HD2 gel station and saved as TIFF files.

RT-PCR specificity of the intron-spanning primers was confirmed by two methods. DNA-only reactions used the same reaction conditions but 50-100 ng of zebrafish genomic DNA instead of total RNA as a template. RT-minus reactions amplified the same amount of total RNA using PCR reagents alone (JumpStart RedTaq PCR Reagent; Invitrogen), without reverse transcription. For our intron-spanning primers, DNA-only reactions showed the larger bands expected from genomic DNA contamination; RT-minus reactions for these primers showed no products (not shown).

Glycol methacrylate sectioning and staining

Freshly dissected adult barbel tissues were fixed in 4% paraformaldehyde-phosphate-buffered saline (PF-PBS; pH 7.6) at 4°C overnight or at room temperature for 1-2 hours. After several rinses in PBS, tissues were dehydrated gradually over several hours to 70% ethanol and stored in the cold until use. For embedding, the specimens were dehydrated to 100% ethanol and infiltrated with cold-polymerizing glycol methacrylate (GMA; Technovit 7100; Electron Microscopy Sciences, Warrington, PA). Precise orientation of the specimens was obtained by pre-embedding in smaller blocks of resin (De Smet et al., 2004; Leroux et al., 2007). Infiltrated barbels were laid horizontally in a small amount of GMA embedding solution in shallow rectangular silicone molds, covered with Parafilm to exclude air, and polymerized overnight at room temperature. These blocks were then embedded with the long axis vertical in a 1.5-ml Eppendorf tube filled with additional GMA, followed by a second overnight polymerization.

For sectioning, the Eppendorf tube filled with solidified GMA was clamped in a vice, and the distal 4-5 mm of the plastic was trimmed off with a high-speed rotary tool (Dremel). Under magnification, the polymerized GMA was hand-trimmed with a razor blade so that the block face approached the specimen. The tube was then clamped in the chuck of an American Optical 820 rotary microtome, and 3-4- μ m sections were cut on a tungsten-carbide blade. Sections were collected singly, floated on a room-temperature bath of distilled water, and mounted in groups of 10-12 on cleaned, coated glass slides (Color-frost Plus; Fisher Scientific, Fair Lawn, NJ). The freshly mounted sections were dried on a 60°C hot plate for 2 hours and stored desiccated at 4°C until use.

Immunohistochemistry on whole mounts and sections

Whole mounts—Whole-mount staining of developing and adult barbels followed a recent protocol developed for newt spinal cords (Zukor et al., 2010). Juvenile heads (30-48 days postfertilization) and adult barbels (3-12 months postfertilization) were fixed in ice-cold Dent's (4 parts methanol:1 part dimethyl sulfoxide [DMSO]) for 4 hours at room temperature or overnight at 4°C . After dehydration to 100% methanol, the tissue was stored at -20°C for up to several months. As a positive control for myelinated axons, whole zebrafish larvae at 4-5 dpf were similarly fixed, stored, and later included in the antibody reactions. Negative controls for the primary antibodies included using these antibodies preabsorbed against 4-5 dpf embryos, which greatly decreased or abolished all signals; negative controls for the secondary antibodies included barbels incubated without primary

antibodies, which showed minimal background staining (data not shown). All steps were carried out on a rocking platform. Fixed tissues in methanol were rehydrated to PBS + 0.5% Triton-X (PBS-TrX), then blocked 2-4 hours in PBS-TrX + 2% DMSO + 10% normal goat serum. After blocking, primary antibody was applied in PBS-TrX + 2% DMSO + 1% normal goat serum at 4°C overnight.

After overnight incubation, the tissues were washed six to eight times in PBS-TrX, then incubated for 2-3 hours at room temperature with either anti-mouse or anti-rabbit fluorescent secondary antibodies (anti-mouse Cy3 or DynaLight anti-rabbit 488; Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in PBS-TrX + 2% DMSO + 1% normal goat serum. Double staining was accomplished by incubating first with anti-MBP and detecting it with green, followed by anti-tubulin and detecting it with red. After all staining steps were complete, the tissues were washed five or six times in PBS, immersed in a 1:1 solution of glycerol:PBS plus DAPI (0.0025 g/ml), rinsed three times in glycerol:PBS, and mounted on glass slides for microscopy. Fluorescent images were obtained on a conventional Nikon 50i fluorescent and/or a Leica SP3 confocal microscope.

Sections—Slides carrying dried GMA sections were placed directly in PBS + 0.1% Triton-X (PBS-TrX; pH 7.6) for 5-10 minutes, followed by a 10-minute treatment with proteinase K (10 µg/ml) in PBS-TrX at 37°C in a humid chamber. The slides were washed, blocked, and incubated with the same solutions and reagents as described above, minus the addition of DMSO. Primary antibody incubations were carried out in a dark, humid chamber overnight at 4°C, using Parafilm coverslips to spread reagents evenly. On the next day, slides were rinsed three times in PBS-TrX for 10 minutes each, then incubated for 2 hours at room temperature with the appropriate secondaries. After three rinses in PBS, the tissue sections were mounted in 50:50 glycerol:PBS with DAPI as a counter-stain, then stored at 4°C in the dark until imaging.

Primary antibody characterization

The mouse monoclonal anti-tubulin antibody was obtained from Sigma (T7451) and has been extensively characterized (*JCN* Antibody Database). It was used at 1:1,000-1:2,000 for this study. The other primary antibody was a polyclonal rabbit anti-zebrafish myelin basic protein (anti-MBP) kindly donated by Drs. Clare Buckley and William Talbot. This reagent has the ZFIN antibody identifier ZDB-ATB-111104-1 and was used at 1:50-100 for this study. The antibody was generated commercially (Sigma Genosys), the immunogen being a zebrafish-specific MBP peptide (CSRSRSPPKRWSTIF) conjugated to keyhole limpet hemocyanin (KLH) via m-maleimidobenzoyl-N-hydroxysuccinimide (MBS; Lyons et al., 2005). To validate the tissue visualization of myelin in this study, we tested this antibody by whole-mount staining of 4-5-days-postfertilization zebrafish embryos; these yielded staining patterns consistent with known myelinated tracts in the embryonic zebrafish CNS (not shown). We additionally tested this antibody on GMA sections of adult zebrafish optic tectum fixed and stained as described above. These sections showed expected staining of known myelinated regions (see Fig. 3C,D). All antimyelin staining patterns detected for barbel tissue were closely associated with the tracts of adjacent axons, as detected by anti-tubulin staining. The only MBP staining not closely associated with axons was seen in some cells of the epithelial taste buds, possibly the “type I” cells (glial-type cells) of this organ (see Fig. 5G).

Transmission electron microscopy

Regenerating maxillary barbels and their contralateral normal controls were fixed by immersion in cold 2.5% paraformaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences) for 3-4 days in a 4°C refrigerator. After three rinses

in cacodylate buffer, they were transferred to 2% osmium tetroxide in 0.1 M sodium cacodylate for 1.5 hours, rinsed three times with distilled water, then stained en bloc with 3% uranyl acetate for 1 hour. Samples were then dehydrated in a graded ethanol series (50-100%), embedded in epoxy resin, and cut on a Leica UC6 ultramicrotome. For light microscopy, 1- μ m-thick sections were stained with toluidine blue O. For transmission electron microscopy (TEM), 70-nm sections were mounted on 200-mesh copper grids and poststained with uranyl acetate and Reynolds lead citrate. Sections were examined in a JEOL JEM 1220 and/or a FEI Tecnai Spirit G2 transmission electron microscope.

Image processing and analysis

Images were adjusted in Adobe Photoshop for brightness and contrast. To obtain complete high-resolution cross-sections of the maxillary barbel, multiple transmission electron micrographs were assembled into composites. Each frame carried its own embedded micrometer scale, ensuring that all images were kept at the same magnification. To compare normal and regenerated axons quantitatively in these images, we used Image J (Rasband, 1997-2012) to measure all visible ventral axons in 3 normal and 3 regenerated maxillary barbels (28 days postsurgery), respectively. Axon areas were measured by using the freehand tool to outline the light gray portion of the cytoplasm within the axoplasm membrane. Nonmyelinated and myelinated axons were distinguished by inspection. For myelinated axons, myelin thickness was measured by drawing a line segment through a region of compact, well-preserved myelin for each axon, typically at the 12 o'clock position. Axon diameter was back-calculated from axon area, assuming that each axon was a perfect circle:

$$\text{Axon diameter} = 2 \cdot \left(\sqrt{A/\pi} \right).$$

Fiber diameter was calculated as (axon diameter + [2 myelin thickness]). g-ratio, a measure of myelin thickness with respect to axon size, was calculated by dividing axon diameter by fiber diameter. Two observers (A.K.H., E.E.L.) performed the digitization, obtaining similar results. Graphing and statistical analyses were performed in GraphPad Prism. Data distributions were assessed for normality by using the D'Agostino and Pearson omnibus normality test. Percentages of myelinated axons were compared by using the two-sample, two-tailed z-test of proportions. Differences in central tendency were tested by using the nonparametric equivalent of the two-tailed, two-sample t-test (Mann-Whitney U). Equality of continuous distributions was tested by using the two-sample, two-tailed Kolmogorov-Smirnov test (http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). To correct for multiple, nonindependent tests while maintaining an overall type I error rate of approximately 5%, we adjusted our alpha value downward by using the Bonferroni correction, namely, (0.05/8 tests performed) = $P < 0.006$ to declare statistical significance for each test (Sokal and Rohlf, 2011).

RESULTS

Maxillary barbel axons express *mbp* and are wrapped in thick myelin sheaths

To determine whether maxillary barbel nerves were myelinated, we used RT-PCR to detect transcripts of *mbp*. *mbp* is strongly expressed in the zebrafish CNS and PNS from embryonic day 5 onward, making it a common molecular marker of myelinating activity in the zebrafish nervous system (Buckley et al., 2010; Pogoda et al., 2006). Two previously identified alternatively spliced *mbp* transcripts were detected in both the normal adult barbel shaft and the distal tips of regenerating adult barbels at 10 and 14 days postsurgery (Fig. 2).

To examine the distribution of myelin in normal maxillary barbel neurons, we prepared thin (1-4 μm) plastic sections of adult tissue for light microscopy and immunohistochemistry. Slides stained with toluidine blue O revealed many small (1-2 μm), dark rings in the dorsal and ventral neural regions (Fig. 3A, dn and vn), suggesting abundant myelination. To confirm that these areas contained myelin, we probed tissue sections with a zebrafish-specific anti-MBP primary antibody and a fluorescent secondary. The result was a high-contrast pattern of minute fluorescent punctae clustered within the axon-rich ventral region (Fig. 3B). Smaller clusters of punctae were observed in the dorsal nerve region and at the bases of epithelial taste buds. The best resolution of myelin in situ was achieved by preparing ultrathin sections for TEM. These samples confirmed that most axons of the maxillary barbel are indeed thickly myelinated, with numerous tightly packed lamellae (Fig. 4).

Early barbel buds are innervated but lack myelination; myelination proceeds from proximal to distal and from ventral to dorsal to establish the adult pattern

Having detected abundant myelination in the adult maxillary barbel, we sought to discover when this myelination becomes established during development. To examine the early stages of barbel development, we used whole-mount immunohistochemical techniques to stain axons and/or myelin in a series of juvenile zebrafish.

As previously reported (LeClair and Topczewski, 2010), the maxillary barbel bud is innervated from its very first extension with a central tract of fibers descending from the facial nerve (cranial nerve VII). Anti-acetylated tubulin staining confirmed this pattern (Fig. 5A-F); however, at early juvenile stages (<0.3 mm barbel length), myelin within the barbel bud was undetectable (not shown). Only barbels that had grown past the edge of the maxilla, protruding several hundred micrometers (>0.5 mm barbel length), showed proximal myelination in the large (ventral) nerve tract (Fig. 5G,H). Myelin staining was weak or absent distally. The ventral nerves, which invade first, were myelinated prior to the dorsal nerves, which invade later (not shown).

Although developmental myelination of the maxillary barbel nerves lagged behind the extension of axons, adult maxillary barbels stained with the same antibodies showed abundant myelination in most areas where axons occur (Fig. 6). In whole mounts, almost all fibers in the ventral and dorsal nerve tracts appeared myelinated along most of their length. However, individual nerve fibers within these tracts, small fibers crossing between the dorsal and the ventral tracts, or sections of otherwise myelinated nerves could be unmyelinated.

Early-regenerating axons are nonmyelinated or have disorganized myelin, and collagenous matrix and fibroblasts predominate

We next wanted to know whether the adult pattern of extensive myelination was restored during maxillary barbel regeneration and, if so, in what period of time. As previously shown, regenerating maxillary barbels have a normal epithelium but an abnormally thick, matrix-rich core that resembles scar tissue (LeClair and Topczewski, 2010). We thus speculated that this matrix might be a barrier to regenerating axons and/or migrating Schwann cells. To study axon regeneration in this context, we collected regenerating maxillary barbels at 10 days postsurgery and prepared TEM sections just distal to the amputation plane (Fig. 7). The following observations are based on five or six sections per barbel from eight barbels collected at this time point.

Unlike a tightly organized normal barbel (see, e.g., Fig. 3A), early-regenerating barbel tissue appeared patchy, with many voids or holes in both the ectodermal and the mesodermal

layers (Fig. 7A). Under TEM, these electrolucent voids were filled with loose collagen fibers, microtubules, and/or fragmentary cell membranes (Fig. 7B,C). There were no large, intact axons. A few small intact axons were observed, most of which were unmyelinated. The most myelin observed was eight to 10 wraps of loose myelin around a disorganized core (Fig. 7D). Thick, dark sheaths of myelin were not present. Schwann cells were not evident, nor were there any obvious inflammatory cells. The most abundant cells in the mesodermal compartment at this stage were large, irregular cells full of rough endoplasmic reticulum and Golgi (Fig. 7E). This is typical of fibroblasts, a major source of tissue collagen. Consistent with this interpretation, these fibroblastic cells surrounded and in some cases were cytoplasmically continuous with extracellular collagenous fibers, suggesting cellular secretion and/or absorption. Numerous collagen bundles were observed in cross, oblique, and longitudinal sections (Fig. 7F), indicating a mixed or random orientation of fibers in the regenerating tissue.

Late-regenerating axons are altered in position, but axon morphology and myelination are restored

Because few intact axons were observed in distal barbel tissue at 10 days postsurgery, we repeated our surgical experiments, this time allowing the tissues to regenerate for 28 or 70 days, respectively (Fig. 8A). Results from both time points were similar. For brevity, only the 28-day results are shown.

Regenerating barbels at 28 days appeared similar to the original appendages, having grown out several millimeters (see, e.g., Fig. 1B). In contrast to the patchy, fluid-filled tissue seen at 10 days postsurgery, these appendages had a largely normal histology. Most features of the normal adult barbel were restored, including a thick, glandular epithelium with numerous taste buds and goblet cells; a simple vascular system of two or three endothelially lined fluid-filled tubes; and a large, centrally located nerve bundle (Fig. 8B,C). Consistent with our previous descriptions based on paraffin histology, 100% of the regenerated barbel cores had a disorganized mesodermal matrix, which was expanded into a ring around the central soft tissues. Unlike the loose collagen bundles surrounded by fibroblasts, this matrix was dense and largely acellular; occasional nucleated cells were embedded within. In regenerated barbels, the major nerve trunks were also repositioned. Unlike the case in normal barbels, we consistently observed one large bundle of axons ventrally and few or no axons dorsally.

Higher magnification of the ventral bundle showed six to eight smaller nerve fascicles, each with 10-20 intact axons (Fig. 8D). Cross-sections through these axons showed well-organized electrolucent axoplasms packed with abundant microtubules. Most of the regenerated axons were thickly myelinated, in close association with Schwann cells (Fig. 8E).

Regenerated axons are similar to normal axons in fiber type and caliber but have slightly reduced myelination

To compare normal and regenerated axons further, we digitized all visible ventral axons from complete cross-sections of three normal and three regenerated (28 days postsurgery) adult maxillary barbels. Nerves outside of the dermal region, such as at the bases of the tastebuds, or within the epidermis, were not examined. Over 900 axons were measured for axoplasm area (in μm^2), and, if the axons were myelinated, we measured the thickness of the corresponding myelin layer (in μm). The absolute difference in axon number was not considered important; adult fish were not strictly size matched, and larger individuals, such as females, would be expected to have more axons (Bailey et al., 2007). Axon number would also be expected to vary proximodistally along the appendage; small variations in

section placement, therefore, might sample different numbers of axons. Rather, we sought to compare percentages (for categorical variables) or distributions (for continuous variables) between all normal and all regenerated axons, respectively.

Normal adult barbels had between 122 and 223 ventral axons, an almost twofold difference between individual samples (Table 2). Despite this range of axon number, the proportion of myelinated fibers in each normal appendage was much less variable, between 59% and 65%. A test of proportions on the pooled, within-group data showed that the percentage of myelinated fibers did not differ between normal and regenerated tissue ($316/499 = 63.4\%_{\text{normal}}$ vs. $306/483 = 63.3\%_{\text{regenerated}}$; two-sample, two-tailed z -test = 0.76, $P = 0.93$).

In both normal and regenerating appendages, myelinated axon cross-sectional areas were strongly skewed rightward, indicating many small but few large axons of this subtype (Fig. 9A,B; D'Agostino-Pearson omnibus normality test, $P < 0.0001$). Most myelinated axons were less than $2 \mu\text{m}^2$. Unmyelinated axons were smaller in caliber, less than $1 \mu\text{m}^2$, and were also nonnormally distributed ($P < 0.0001$). There were no large, unmyelinated axons. The calibers of myelinated and unmyelinated fibers were overlapping but not identical. Although they were smaller, all unmyelinated axons were within the size range of myelinated axons.

The median cross-sectional areas of myelinated and unmyelinated axons were similar between normal and regenerated tissue, indicating that, after 28 days, the typical caliber of each type of axon was restored (Mann-Whitney U; myelinated_{normal} vs. regenerated, 0.72 vs. $0.75 \mu\text{m}^2$, $U = 44788$, $P = 0.11$; unmyelinated_{normal} vs. regenerated, 0.22 vs. $0.27 \mu\text{m}^2$, $U = 14719$, $P = 0.13$). The cross-sectional areas of each fiber type were also not significantly different when compared by using a highly sensitive test for equality of continuous distributions (Kolmogorov-Smirnov; myelinated axon areas_{normal} vs. regenerated, $D = 0.09$, $P = 0.14$; unmyelinated axon areas_{normal} vs. regenerated, $D = 0.11$, $P = 0.19$). A cumulative frequency plot comparing the crosssectional areas of normal and regenerated myelinated axons confirmed almost identical curves along the same x-axis scale (Fig. 9E).

Median myelin thickness was slightly less in regenerated tissue relative to controls, a highly significant result (Fig. 9C vs. D; 0.11 vs. $0.13 \mu\text{m}$, Mann-Whitney $U = 34,567$; $P < 0.0001$). A significant difference was also detected when the two groups were compared for equality of continuous distributions (Kolmogorov-Smirnov; $D = 0.25$, $P < 0.0001$). This difference was further confirmed by a cumulative frequency plot of the two distributions, showing that the myelinated axons in regenerated maxillary barbels have thinner myelin in almost all size classes (Fig. 9F). Proportionally, these regenerated axons have myelin sheaths about 15% thinner, on average, than normal axons, the absolute between-group median difference being approximately $0.02 \mu\text{m}$ (20 nm). Stated another way, regenerated maxillary barbel axons restore, after 28 days, approximately 85% of normal myelin levels.

Another common measure of myelination is the g-ratio, or the inner axonal diameter divided by the outer axonal ("fiber") diameter. Lower g-ratios indicate thicker myelin. The g-ratios for normal and regenerated axons were both negatively skewed (D'Agostino and Pearson omnibus normality test, $P < 0.0001$), indicating a small number of "hypermyelinated" axons in each population. Unsurprisingly, given the slightly thinner myelin detected in regenerated axons, the g-ratios of these regenerated fibers were slightly higher relative to controls (Fig. 10A vs. B; Mann Whitney U, g-ratio_{normal} = 0.79 vs. g-ratio_{regenerated} = 0.83; $U = 32,530$, $P < 0.0001$). There was, however, considerable variation both within and among groups. Notably, the g-ratio range for normal axons was quite large (0.55-0.91), and almost identical to the g-ratio range for regenerated axons (0.57-0.96). Scatterplots of g-ratio vs. axon diameter also revealed considerable overlap between the axons in the two groups (Fig. 10C

vs. D). There was a similar, slightly curvilinear relationship in each plot, suggesting that smaller axons tend to be more thickly myelinated than larger ones. This relationship was quite noisy, however, particularly in normal fibers, for which a larger scatter was observed.

Regenerating maxillary barbels express multiple promyelinating transcription factors

To confirm that the myelin sheaths that we observed in regenerating barbels represented de novo myelin synthesis, we isolated total RNA from distal barbel tissue 28 days postsurgery (Fig. 11A) and used RT-PCR to screen for multiple promyelinating transcription factors. This analysis strongly amplified all four targeted transcription factors, *sox10*, *pou3f1*, and *egr2a/b*, from distal regenerating barbel tissue (Fig. 11B). Positive (RT⁺) and negative (no RNA) controls for a typical housekeeping gene (β -actin) gave expected results of one band and no band, respectively. The entire experiment was repeated on a second, independently isolated pool of regenerating barbel tissue, producing similar results (not shown).

DISCUSSION

How nerves regenerate is still a puzzling question. After injury, surviving axons must extend through an altered environment of cells, fluid, and matrix. To remyelinate these axons, Schwann cells must proliferate, migrate, and colonize each fiber, wrapping it with an amount of myelin appropriate to its size. Solving this puzzle within the context of an adult organism depends on experimentally tractable systems in which we can observe how axons and glia coregulate to reestablish myelination, conduction, and sensation. The maxillary barbel is a novel tissue system in zebrafish that offers several avenues for the study of peripheral nerve myelination. Adult barbels are externally accessible, optically transparent, and regenerate rapidly. Surprisingly, barbel innervation in zebrafish has not been closely studied, but these organs are likely important for zebrafish sensory biology and may provide new means to study how vertebrate peripheral nerves respond to injury and regrow into traumatized tissues.

Previous investigations in our laboratory had tracked the development and regeneration of maxillary barbel axons but had not established whether these axons were myelinated before or after injury. In this study, we detected a common myelin-associated transcript, *mbp*, in both normal and regenerating zebrafish barbels. This constitutive expression is consistent with recent reports from mice indicating that myelin-associated genes not only are expressed during embryogenesis but are required for adult myelin maintenance (Decker et al., 2006). Myelin was confirmed in situ by antibody staining and quantified by transmission electron microscopy. In the normal adult zebrafish sampled for this study, approximately 60% of maxillary barbel axons had myelin lamellae. Approximately 40% of the axons were unmyelinated, and these were always the smallest caliber axons. Because of the difficulty of distinguishing extremely small axons from other cellular features in the micrographs (e.g., thin projections of Schwann cells), our measurements may misestimate the proportion of unmyelinated axons. Conversely, some myelinated axons may be misclassified because the axon myelination in the barbel is not continuous but can have gaps; in a single section, such an axon would appear to lack myelin. Under the assumption that these gaps are not experimental artifacts, their presence may reflect the normal, stable, physiological state of intermittent myelination, which has been described for other vertebrate axons (Smith et al., 1985).

There are few electron micrographic studies of fiber types of fish cranial nerves and even fewer reports on the cranial nerves specific to barbels or related sensory appendages (Andres, 1993; Bailey et al., 2007; Bartheld and Meyer, 1985; Fox, 1999; Joyce and Chapman, 1978; Kotrschal et al., 1993). Although such nerves vary in placement and function, most contain a similar mixture of fiber types and sizes. Studies of a tooth-related

lower jaw nerve (the trigeminal alveolar) in the cichlid *Tilapia mariae* documented 51-76% myelinated fibers, depending on body size, and many myelinated axons less than 1 μm in diameter (Hildebrand et al., 1988; Tuisku and Hildebrand, 1992). A developmental survey of the recurrent facial nerve in the catfish *Ictalurus punctatus* reported that approximately 55% of axons were myelinated and that this proportion remained roughly constant over a seven-fold increase in body size (Finger et al., 1991). Small fish of this species had the greatest number of small myelinated fibers (<0.4 μm in diameter); however, the lower limit of myelinated fiber size increased with increasing standard length. Our observations of approximately 60% myelinated fibers in maxillary barbel axons and many extremely small myelinated axons within this population are consistent with these reports. Assuming that the percentage of myelinated fibers is positively correlated with body size, our data, which are based on adult zebrafish only (~2.5-3.0 cm standard length, <1 year old), likely represent close to the maximum myelination percentage for axons of this appendage. Further ontogenetic changes, however, cannot be ruled out. It has been reported that, within highly inbred mice, the percentage of myelinated axons in the auditory nerve varies by individual, strain, and age (Little and Heath, 1994). It is therefore possible that other strains or stages of zebrafish may show axon profiles different from those reported here.

In contrast to previous studies of fish facial and lateral line nerves reporting a bimodal distribution of nerve fiber diameters, e.g., small and large (Finger et al., 1991; Thomas, 1956), we found in the maxillary barbel a unimodal distribution for both myelinated and unmyelinated fibers. Almost all of the myelinated fibers are less than 2 μm^2 ; in each barbel, there are only two or three larger, “giant” axons. Unmyelinated fibers are even smaller, with most less than 1 μm^2 . Thus, even the largest axons in the maxillary barbel are within the small range as previously reported for other fishes. This is not unexpected, because zebrafish are smaller fish. Also, maxillary barbel axons are anatomically more distal compared with some other cranial nerve regions sampled in earlier studies; predictably, then, barbels contain only very small fibers that have branched from more proximal nerves.

Large axons are inherently fast conducting; smaller axons can increase conduction velocity with myelination. It was once thought, on theoretical grounds, that there was a lower limit to myelination and that myelin would slow or even prevent conduction in very small (<1 μm diameter) axons (Douglas and Ritchie, 1962). Subsequent studies, however, found no decreased conduction in myelinated fibers of this size (Ritchie, 1982). The absence of a lower limit to myelination seems to be upheld in the maxillary barbel, which has both extremely small myelinated and nonmyelinated axons. In addition to small size, the considerable length of barbel axons may make myelin sheathing more critical. Long barbels have evolved independently in many species of fishes as adaptations for sensing food, finding mates, and avoiding predators (Bailey et al., 2007; Kasumyan, 2011; Kiyohara et al., 2002; Kotrschal, 2000; for review see Collin, 2007). Barbel lengths vary but range from ~10% of body length in zebrafish up to 50-200% of body length in other fishes. Therefore fast conduction of certain signals from these appendages may be particularly in demand.

The significance of the unmyelinated axons within the maxillary barbel is unclear. In both normal and regenerated barbels, the caliber of unmyelinated fibers is entirely within the range of the myelinated fibers. Therefore, axon size does not predict myelination. Two explanations, not mutually exclusive, have been proposed (Finger et al., 1991). One is that unmyelinated axons represent young, growing axons that are not yet mature or fully wrapped. The other is that unmyelinated axons represent a specialized neural subpopulation, possibly innervating solitary chemosensory cells (SCCs) or ending freely within the epidermis. Finally, it is possible that some of the unmyelinated fibers in the maxillary barbel are sympathetic efferents. The maxillary barbel has no intrinsic musculature; however, small efferents might conceivably regulate blood flow in the microcapillaries of this appendage.

Because we did not trace the path of individual neurons, however, the destinations and connections of these different fiber types are not known.

In the traditional classification of mammalian peripheral nerve fibers, small, unmyelinated axons are termed “C-fibers” because they contribute a low, slow peak (the C-elevation) to a compound action potential, after the faster A and B elevations (Kasumyan, 2011). Judged from their size, distribution, and frequent association with non-myelinating Schwann cells, the unmyelinated axons in the zebrafish maxillary barbel are consistent with such fibers. Historically, this morphological class of neurons was considered to correspond to a particular functional class, specifically nociceptors. More recently, it has been thought that the functional roles of C-fibers may be far more complex, transmitting heterogeneous and nonnoxious stimuli such as pressure or temperature (Douglas and Ritchie, 1962). It is thus possible that the abundant unmyelinated fibers in the zebrafish maxillary barbel allow the organism to sample its environment, albeit slowly, for multiple baseline stimuli. The slightly larger, myelinated maxillary barbel axons might correspond to the classic alpha-delta, or A δ , fibers. Although the slowest of the A fibers, these axons in mammals conduct 10-20 times more quickly than C-fibers, providing more rapid reports from the periphery to the center. In the absence of electrophysiological recordings from zebrafish barbels, however, assigning specific functions or conduction velocities to these fiber types would be premature.

Unlike the zebrafish caudal fin, which regrows a morphologically accurate replacement no matter how many times it is amputated (Azevedo et al., 2011), maxillary barbels respond to amputation with excess fibrous connective tissue that fills the central core (LeClair and Topczewski, 2010). This affects the entire regenerate distal to the amputation plane, yet axons successfully navigate this matrix to innervate the distal tip. Although we hypothesized that this additional matrix might inhibit the Schwann cell movements necessary to remyelinate axons, this was not observed. Instead, regenerated maxillary barbels restored both myelinated and nonmyelinated fibers to normal frequencies and cross-sectional areas within 4 weeks and myelin thickness to ~85% of normal levels within the same interval. The simplest model for the restoration of axon subtype and cross-sectional area would be if each severed axon maintained its preamputation set point for both myelination and size. However, because we sectioned only within the distal, regenerating tissue at 28 days, we cannot correlate the fibers in the stump and the regenerate. The remyelination response of maxillary barbel axons appears robust; however, because only one time point was sampled for morphometric measurements, we do not know whether the slightly thinner sheaths observed around regenerating axons represent a temporary or a permanent deficit. The functional difference that might be caused by the absolute difference in myelination is not known.

The morphological course of maxillary barbel regeneration is similar to that reported for zebrafish optic nerves (Nona et al., 1992, 1998). Specifically, optic nerve crush in vivo causes demyelination and macrophage scavenging of cell debris, followed by axon extension, Schwann cell migration, and the re-expression of myelin markers. This occurs in 40-50 days. In maxillary barbel regeneration, there is little or no myelination at 10 days but extensive restoration within 28 days. Although the contexts and lesions are quite different (CNS vs. PNS, crush vs. amputation), adult Schwann cells are presumably active in both. We confirmed this in the maxillary barbel by detecting, at 28 days, robust expression of *sox10*, *pou3f1*, *egr2a*, and *egr2b* in distal regenerating tissue. All of these transcripts are highly specific for Schwann cell-mediated myelination, so we infer that adult barbel regeneration recapitulates, at least in part, the molecular program of myelination in embryos. This suggests that rapidly regenerating barbel tissue could be used in a relatively short experimental interval to study the local molecular pathways that restore PNS myelin.

The amount of myelination relative to fiber caliber is considered critical for nerve conduction and is most commonly measured by g-ratio. Using both electrical and volume constraints, Chomiak and Hu (2009) recently modeled optimal myelination for CNS neurons, deriving a g-ratio of ~0.77. This means that, for any myelinated axon, the axon diameter should be 77% of the fiber diameter. With adjustment for the shrinkage that occurs in fixed tissue, this corresponds to a measured g-ratio that is slightly higher (~0.76-0.81; Chomiak and Hu, 2009). The median g-ratios for normal and regenerated maxillary barbel axons observed in this study, 0.79 and 0.83, respectively, are at the upper end of this theoretically optimal range. In regenerated barbels, the median g-ratio was slightly higher relative to normal controls, indicating somewhat thinner myelination at the time point measured. Although a completely normal level of myelination was not reached by 28 days postsurgery, the fact that zebrafish maxillary barbels consistently remyelinate several hundred small axons to ~85% of normal levels suggests that there is a cellular demand for myelination in this appendage, which may be regulated to approach a physiological optimum. The endogenous mechanisms that halt myelin synthesis at an appropriate level are not well understood. Recently, several pathways have been described that can cause hypo- or hypermyelination phenotypes in mammalian development, regeneration, or both. These include neuregulin-1/erbB signaling (Fricker et al., 2011; Michailov et al., 2004; for review see Nave and Salzer, 2006) and the membrane-associated enzyme beta-secretase 1 (BACE1; Hu et al., 2008). Other influences on peripheral nerve regeneration include extracellular matrix proteins, neurotrophic factors, and circulating hormones (for review see Chen et al., 2007), all of which might be explored by using the corresponding systems in zebrafish (see, e.g., Lyons et al., 2005; Rojas-Muñoz et al., 2009).

The ZMB is an attractive system for studying myelination because of its small size, rapid growth, and optical transparency. It presents the challenge, however, of detecting some of the smallest peripheral axons in the organism. For axons of this size, TEM will likely remain the gold standard for myelin detection and morphometric analysis. A recent study in mouse auditory nerve, however, combines fluorescent immunohistochemistry for MBP with high-resolution confocal microscopy on standard paraffin sections, suggesting an optical-only approach (Xing et al., 2012). Future studies of maxillary barbel myelination might include a more detailed survey of cell behavior and gene expression in regenerating barbel tissue, including direct observation of Schwann cells and screening for the positive and negative regulators of myelination previously described. Once identified, these cells and molecules might be modified *in vivo* by many of the genetic and molecular tools available in zebrafish. Existing transgenic lines with alterations in embryonic myelination could, if viable, be studied as adults to determine whether the same pathways affect remyelination after injury or disease. Finally, the growing suite of neurophysiological and behavioral assessments available for this species could address how normal and regenerated barbels execute their sensory functions in the context of the organism and its environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Anatomical overview of adult zebrafish maxillary barbel (ZMB) regeneration. **A:** Morphological sequence of ZMB regeneration from 10 to 28 days postamputation. Proximal amputation (dotted line) induces regeneration of a new appendage (black). Barbel regeneration is highly variable; the length of the regenerated tissue is approximate. **B:** Gross morphology of a regenerated maxillary barbel after 28 days at 28°C. The amputation plane (dotted line) is marked by the blunt stump of the central rod of connective tissue, which does not regrow. nb, Nasal barbel; mb, maxillary barbel.

Figure 2.

Normal and regenerating maxillary barbels express alternatively spliced transcripts for myelin basic protein (*mbp*). **Top:** Equal amounts of DNase-treated total RNA were analyzed by RT-PCR for the presence of *mbp*, a common marker of zebrafish myelination. The primers used amplify a doublet, indicating two alternatively spliced transcripts (see Buckley, 2010). Wild-type zebrafish embryos 5 days postfertilization (5 dpf) express both transcripts (+ = positive control). Similar bands were detected in normal adult barbels (norm) and the distal tips of regenerating barbels at 10 and 14 days postsurgery (10 dps and 14 dps). A parallel reaction with water as the template (H₂O, negative control) gave no product.

Bottom: All RNA lanes amplified a single band for β -actin. The starting amounts of total RNA were identical to those in the top panel.

Figure 3.

Myelination of the deep nerve tracts in the zebrafish maxillary barbel. Barbel tissues were collected from adult wild-type AB zebrafish and prepared as thin cross-sections for light microscopy. **A:** A thin (1 μm) plastic section of a normal barbel was stained with toluidine blue O. Myelinated axons with dark, ring-like sheaths appear in the dorsal nerve (dn) and ventral nerve (vn) areas surrounding the central rod of connective tissue (cr). **B:** A similar plastic section stained with DAPI (blue) and an antibody against myelin basic protein (MBP; green). Numerous bright punctae overlay the dorsal and ventral populations of neurons.

C,D: Validation of the anti-MBP primary antibody on sections of zebrafish optic tectum. **C:**

DAPI stain (blue), showing nuclei of the tectal layers. D: DAPI merged with anti-MBP (green). Immunostaining was observed in the expected layers of the tectum, specifically the stratum opticum (so) and stratum album centrale (sac). Positive staining in these layers has been used to validate other myelin-specific antibodies in zebrafish (compare Fig. 1 in Bai et al., 2011). sm, Stratum marginale; so, stratum opticum; sfgs, stratum fibrosum et griseum superficiale; sgc, stratum griseum centrale; sac, stratum album centrale; spv, stratum periventriculare.

Figure 4.

Transmission electron microscopy (TEM) of maxillary barbel myelination. Barbel tissues were collected from adult wild-type AB zebrafish, embedded, and cut at 70 nm. To improve focus, A, B, and D were enhanced with a digital filter (Unsharp Mask) in Adobe Photoshop. **A:** Overview of the axon-rich ventral area in a normal adult maxillary barbel. Multiple small (1-2 μm) myelinated axons are surrounded by accompanying Schwann cells. **B:** In each barbel, there are several 'giant' axons. **C:** High-magnification TEM showing a single barbel axon (a) with numerous closely packed myelin lamellae (m). The axoplasm is full of smaller, grayish microtubules. **D:** Normal barbels also contain bundles of small, unmyelinated axons completely or partially wrapped by the cytoplasm of supporting cells. a, Axon, c, collagen bundle, en, endothelial cell, m, myelin, s, Schwann cell nucleus.

Figure 5.

Development of myelination in the juvenile zebrafish maxillary barbel. Juvenile wild-type AB zebrafish (30-44 days postfertilization) were stained in whole mount to observe the outgrowth of maxillary barbel axons (anti-acetylated tubulin, red) and/or the deposition of myelin (anti-mbp, green). DAPI was used as a nuclear counterstain (blue). The distal tip of each barbel projects rightward. **A-C**: Early maxillary barbel buds (mb, ~150 μm from base to tip) have one ventral nerve bundle that extends to the distal tip (arrows, B,C). At this stage, these axons appear unmyelinated (not shown). **D-F**: More developed barbel buds (~300 μm from base to tip) have more nerve fibers (arrows, E,F) and more projections to the ventral epithelium. Overt myelination is still absent (not shown). The anti-tubulin antibody also labels neuromast organs of the lateral line (arrowheads, E,F). **G**: Juvenile maxillary barbels (~1 mm long) have partial myelination of the ventral, but not dorsal, nerve tracts. Myelination proceeds distally and lags the growing axons. dn, Dorsal nerves; vn, ventral nerves. **H**: Ventral view of a juvenile maxillary barbel (~1 mm long). In this view, the

ventral nerves (vn) are roughly divided into two lateral bundles (arrows, top panel). Only some of these tracts are myelinated (MBP, bottom panel), and myelination extends for only part of the way to the growing tip. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6.

Myelination of mature axons in the adult zebrafish maxillary barbel. Barbel tissues were collected from adult wild-type AB zebrafish and prepared as whole mounts for immunohistochemical staining of acetylated tubulin (AC-TUB; red) and myelin basic protein (MBP; green). This figure shows the midsection of the barbel shaft; proximal is to the left and distal to the right. **A:** The adult maxillary barbel has two populations of nerves, dorsal nerves (dn) and ventral nerves (vn). A few small-caliber fibers run orthogonally (asterisk). **B:** Most axons are myelinated, with thick MBP-positive sheaths; however, some fibers or regions of fibers appear unmyelinated (arrow) or with gaps (arrowhead). **C:** Merged view of the two channels. dn, Dorsal nerves; vn, ventral nerves. A magenta-green copy of this image is provided as Supporting Information Figure 1.

Figure 7.

Ultrastructure of zebrafish maxillary barbel regenerates 10 days postsurgery (dps). Maxillary barbels were amputated from adult wild-type AB zebrafish and allowed to regenerate for 10 days. The regenerating portion of the barbel shaft was fixed and prepared as cross-sections for light and transmission electron microscopy (TEM). **A:** Light micrograph of a 10-dps regenerating barbel stained with toluidine blue O. The epithelial (e) and mesenchymal layers appear patchy, and the central rod (cr) is disorganized. The ventral nerve region (vn) lacks dark rings of myelin (compare Fig. 3A). **B:** Low-magnification TEM of the 10-dps ventral nerve region shows numerous electronlight voids (v) filled with cell debris, surrounded by

fibroblasts (f) and collagen bundles (c). There are few intact axons (a). **C:** Similar magnification of a second ventral nerve region, showing structures as in B. **D:** Magnification of the boxed area in C. The right structure has six to eight loose myelin layers (arrowhead). **E,F:** Regenerates at 10 dps are full of large fibroblastic cells (f) filled with rough endoplasmic reticulum and Golgi, in close contact with cross-cut and longitudinally cut collagen bundles (c). a, Axon; bm, basement membrane; bv, blood vessel; c, collagen bundle; cr, central rod; e, epithelium; f, fibroblast; v, void; vn, ventral nerves. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 8.

Ultrastructure of zebrafish maxillary barbel regenerates 28 days postsurgery (dps). **A:** Maxillary barbels were amputated from adult wild-type AB zebrafish and allowed to regenerate for 28 dps. The barbels were fixed and prepared as cross-sections for light and transmission electron microscopy (TEM). Sections were cut to sample regenerating tissues ~0.5-1.0 mm distal to the amputation plane (far right, dotted lines). **B:** Light micrograph of a 28-dps regenerating barbel stained with toluidine blue O. The epithelium is compact, with regenerated goblet cells (gc) and taste buds (tb). Abundant myelinated axons fill a large ventral nerve trunk (vn) surrounded by a disorganized central rod (cr). Two small blood vessels (bv) carry erythrocytes. **C:** Electron micrograph of a second 28-dps barbel, showing structures similar to those showing in B. **D:** Magnification of the boxed area in C. The

ventral nerve trunk is divided into several smaller bundles, each with 10-15 axons. Most axons appear myelinated, with accompanying Schwann cell nuclei (s). **E**: Magnification of the boxed area in D. Ventral nerve axons are heterogeneous, including large and small myelinated axons and smaller unmyelinated ones (asterisks). bv, Blood vessel; cr, central rod; e, epithelium; gc, goblet cell; tb, taste bud; s, Schwann cell nucleus; vn, ventral nerves; ZMB, zebrafish maxillary barbel; asterisk, unmyelinated axon. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 9.

Distribution of axoplasm area and myelin thickness in normal and regenerated (28 days postsurgery) zebrafish maxillary barbels. Adult wild-type AB zebrafish barbels were prepared for transmission electron microscopy, and 982 axons were measured for cross-sectional area (μm^2) and thickness of myelin (μm), if present. For complete descriptive statistics see Table 2. **A,B**: Pooled, within-group histograms of axon cross-sectional area in normal (A) and regenerated (B) maxillary barbel axons. Myelinated and unmyelinated fiber distributions are interleaved. **C,D**: Pooled, within-group histograms of myelin thickness in normal (C) and regenerated (D) maxillary barbel axons. **E,F**: Cumulative frequency plots for myelinated axon cross-sectional area (E) and myelin thickness (F), comparing normal and

regenerated tissue (solid vs. dotted line). Normal and regenerated barbels have equivalent distributions of axon caliber; regenerated axons have slightly thinner myelin.

Figure 10.

g-ratio analysis of normal and regenerated (28 days postsurgery) maxillary barbel axons. Adult wild-type AB zebrafish barbels (three normal, three regenerated) were prepared for transmission electron microscopy and digitized for morphometric analysis, yielding a g-ratio = (axon diameter/fiber diameter) for each myelinated axon. Larger g-ratios indicate thinner myelin. For complete descriptive statistics see Table 2. **A,B:** Pooled, within-group histograms of g-ratio for normal (A) and regenerated (B) maxillary barbel axons. **C,D:** Scattergrams of g-ratio vs. axon diameter (μm) in normal (C) and regenerated (D) maxillary barbel axons.

Figure 11.

Expression of promyelinating transcription factors in the distal regenerating zebrafish maxillary barbel. **A:** Maxillary barbels from adult wild-type AB zebrafish were amputated proximally and allowed to regrow for 28 days. Only the distal, regenerated tissue was processed for RNA isolation. **B:** Equal amounts of DNase-treated total RNA were split into separate one-step reaction tubes and simultaneously amplified by reverse-transcription polymerase chain reaction (RT-PCR). Primers for the promyelinating transcription factors *sox10*, *pou3f1*, and *egr2a/b* (= *krox20a/b*) all amplified the predicted bands (for product sizes see Table 1). To check RNA integrity, β -actin was amplified simultaneously as a positive control (+). A negative control (-) used the same master mix reagents + β -actin primers, but no RNA.

TABLE 1

RT-PCR Primers and Expected Products

Gene	ZFIN ID No.	Forward primer 5'-3'	Reverse primer 5'-3'	cDNA (bp)
<i>mbp</i>	ZDB-GENE-030128-2	AAGCACCTCTGGACAAAACC	ACGAGGAGAGGACACAAAGC	240, 203
<i>sox10</i>	ZDB-GENE-011207-1	GCTGAACGAGACGGATAAGC	ATTTTCCTCCCTGCAGTTCC	323
<i>pou3f1(oct6)</i>	ZDB-GENE-980526-372	ACAGGCACTTGGTCAGATCC	TTTAGCTGGAGCAGACATGC	347
<i>egr2a(krox20a)</i>	ZDB-GENE-030723-6	GATTTTCATGCGCTGTTTGC	CCCTCTTGACTCCAACCTCC	266
<i>egr2b(krox20b)</i>	ZDB-GENE-980526-283	GAGCTTGCCACAACACTGC	GGGTACTGGGAGTCGATGG	218

TABLE 2

Morphometric Measurements of Zebrafish Maxillary Barbel Axons¹

	No. myelinated, unmyelinated axons (total axons, % myelinated)	Myelin axon area, μm^2 (median, range)	Unmyel axon area, μm^2 (median, range)	Myelin thickness, μm (median, range)	g-ratio (median, range)
Normal barbel					
1	80, 42 (122, 65.5)	0.78, 0.11–3.30	0.29, 0.08–0.99	0.11, 0.04–0.24	0.83, 0.60–0.91
2	95, 59 (154, 61.7)	0.94, 0.25–6.44	0.31, 0.04–2.07	0.14, 0.06–0.33	0.80, 0.65–0.91
3	141, 82 (223, 63.2)	0.55, 0.14–3.00	0.12, 0.02–1.83	0.13, 0.03–0.49	0.75, 0.55–0.90
Regenerated barbel					
1	82, 42 (124, 66.0)	0.81, 0.14–4.93	0.40, 0.10–1.50	0.11, 0.03–0.22	0.81, 0.57–0.95
2	122, 65 (187, 65.2)	0.79, 0.09–2.60	0.26, 0.02–2.11	0.11, 0.04–0.30	0.82, 0.63–0.96
3	102, 70 (172, 59.3)	0.72, 0.15–3.10	0.17, 0.02–0.89	0.09, 0.06–0.16	0.84, 0.69–0.90
All normal (n = 3)	316, 183 (63.4)	0.72, 0.11–6.44	0.22, 0.02–2.07	0.13, 0.03–0.49	0.79, 0.55–0.91
All regenerated (n = 3)	306, 177 (63.3)	0.75, 0.09–4.93	0.27, 0.02–2.11	0.11, 0.03–0.30	0.83, 0.57–0.96

¹Fiber type, axon cross-sectional area, and myelin thickness were measured from transmission electron micrographs of normal (n = 3) and 28 days postsurgery regenerating tissue (n = 3). All fish were 6–12 months old of a wild-type “AB” strain. g-ratio calculations are described in Materials and Methods.