Dynamics of axonal regeneration in adult and aging zebrafish reveal the promoting effect of a first lesion

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Axonal regeneration is a major issue in the maintenance of adult nervous systems, both after nerve injuries and in neurodegenerative diseases. However, studying this process in vivo is difficult or even impossible in most vertebrates. Here we show that the posterior lateral line (PLL) of zebrafish is a suitable system to study axonal regeneration in vivo because of both the superficial location and reproducible spatial arrangement of neurons and targets, and the possibility of following reinnervation in live fish on a daily basis. Axonal regeneration after nerve cut has been demonstrated in this system during the first few days of life, leading to complete regeneration within 24 h. However, the potential for PLL nerve regeneration has not been tested yet beyond the early larval stage. We explore the regeneration potential and dynamics of the PLL nerve in adult zebrafish and report that regeneration occurs throughout adulthood. We observed that irregularities in the original branching pattern are faithfully reproduced after regeneration, suggesting that regenerating axons follow the path laid down by the original nerve branches. We quantified the extent of target reinnervation after a nerve cut and found that the latency before the nerve regenerates increases with age. This latency is reduced after a second nerve cut at all ages, suggesting that a regeneration-promoting factor induced by the first cut facilitates regeneration on a second cut. We provide evidence that this factor remains present at the site of the first lesion for several days and is intrinsic to the neurons.

Schwann cells | axonogenesis | neuromast | sensory system

The potential of adult neurons to regenerate their axons and to reinnervate target organs after injury is not as well understood as early axonogenesis. Whether and how this capability is modified on aging is an even less explored area. In all vertebrates studied so far, neurons of the peripheral nervous system retain the ability to reextend peripheral axons and reestablish functional connections. This ability is thought to involve intrinsic mechanisms of repair as well as extrinsic signals from the local environment, mostly coming from Schwann cells and macrophages (1).

The posterior lateral line (PLL) of fish is a convenient yet unexplored sensory system to address the issue of axonal regeneration throughout adulthood. The PLL comprises a set of superficial mechanosensory organs called neuromasts, which are distributed over the body and tail. Neuromasts are composed of a core of mechanosensory hair cells providing information about the local water flow, surrounded by accessory cells. The afferent neurons innervating neuromasts are clustered in a ganglion posterior to the otic vesicle, and their peripheral axons extend toward the tail, right under the skin. This sensory system is involved in a large repertoire of behaviors (2).

The juvenile PLL of zebrafish comprises four lines of neuromasts that extend at different dorsoventral levels, totaling about 50 organs (3). This pattern is established around 1 mo post-fertilization (mpf) and remains essentially unchanged throughout adulthood, except that each juvenile neuromast gives rise to a number of “accessory” neuromasts through a budding process

(4, 5) that depends on innervation (6). Bud-neuromasts remain closely associated and form dorsoventrally arranged linear clusters, or “stitches” (7). Here we concentrate on the most extensive of the four lines, the ventral one. This line lies originally along the horizontal myoseptum of the embryo and comprises only five neuromasts. More neuromasts develop during larval life, such that the line eventually consists of one neuromast on every intersomitic border, or about 30 altogether (8). The line migrates ventrally to reach its final position at the juvenile stage, and an axonal branch follows each neuromast during this migration.

Axonal regeneration after nerve cut has been demonstrated in the PLL during the first few days of life, leading to complete reinnervation of neuromasts within 24 h (9, 10). Regenerating axons follow either the Schwann cells that ensheath the nerve (10) or the interneuromast cells that extend between consecutive neuromasts (11). PLL nerve regeneration has not yet been addressed beyond the early larval stage.

Here we examine whether the PLL nerve is able to regenerate in adult and aging zebrafish. Our data clearly demonstrate the effectiveness and fidelity of regeneration at all ages studied, from 1 to 15 mpf, although the onset of reinnervation is increasingly delayed with age, thereby linking neuronal aging with a progressive decline in neuronal reactivity to axonal damage. Whenever a second cut is made after complete regeneration, the latency of reinnervation is reduced at all ages, provided the second cut is immediately distal to the first one. We show that although Schwann cells act as guidance cues to help the axons regrow along their original path, they are not involved in this regeneration-promoting effect. We conclude that the promoting effect of a first lesion is mostly caused by an intrinsic, local change occurring in

Significance

The posterior lateral line of zebrafish is a suitable system to study axonal regeneration in vivo because of the superficial location of neurons and targets, allowing one to follow reinnervation in live fish on a daily basis. Here we demonstrate the effectiveness and fidelity of regeneration throughout adulthood. We show that the latency before the nerve regenerates increases with age but is reduced after a second nerve cut at all ages, suggesting that a regeneration-promoting factor induced by the first cut facilitates regeneration on a second cut. We provide evidence that this factor remains present at the site of the first lesion for several days and is intrinsic to the neurons themselves.

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Fig. 1. Patterns of PLL nerve regeneration. (A–D) Nerve regeneration in a mosaic fish in which most PLL neurons are labeled. Nerve branches on somites 14–25 before the nerve was cut (A), 2 d later (B), 3 d later (C), and 6 d later (D). Wallerian degeneration is evident in B and C. Regeneration has reached somite 13, just left of panel B, after 2 d and was complete including the caudal lines after 6 d. Arrows in A point to slight departures from the usual pattern; all departures were faithfully reproduced by the regenerating axons (arrows in D). Asterisks in D point to differences between the original and the regenerated patterns. The cut was done at the level of somite 2 on a 1.5-mpf mosaic fish derived from a wild-type blastula transplanted with cells from a *nbt:dsred* donor blastula. Most or all PLL neurons were fluorescent on the left side of this particular mosaic fish. (Scale bar, 500 μ m.)

the injured axons. Altogether, our results reveal that the zebra-fish PLL is a convenient system to study axonal regeneration in vivo compared with mammalian systems, in which nerve wiring is more complex and less traceable.

Results

Pattern and Dynamics of Reinnervation. We first examined whether the PLL nerve remains capable of regenerating and reinnervating target organs in adulthood, using the *nbt:dsred* reporter line in which all neurons are labeled. In this line, PLL axons are easily visualized at all ages, as they run right under the skin (Fig. S1). Because of higher background fluorescence in the abdominal region, however, we routinely performed the nerve cuts at the level of the anus. We observed that regeneration and reinnervation take place from the onset of adulthood, at 1 mpf, until at least 15 mpf (Fig. S1). Reinnervation proceeds from proximal to distal, with occasional “gaps” that are invariably filled the next day. Irregularities in branch shape are usually conserved on regeneration (Fig. S1 B and C, arrows), although minor changes between original and regenerated patterns may be observed within the stitches (Fig. S1B, asterisks).

We quantified pattern conservation in 17 juveniles at 1 mpf. Branches normally arise at the level of intersomitic borders, but departures from this pattern are occasionally observed. Of 64 irregularities (nerve branches leaving the lateral nerve at least half a somite anterior, or posterior, to the normal branching point) we observed that 51 were reproduced after reinnervation and 13 were lost. Because background fluorescence as well as the intense fluorescence of the spinal cord make this behavior somewhat difficult to document, we generated mosaic fishes by injecting *nbt:dsred* cells into wild-type blastulae to bypass this technical problem. In some of the mosaics, a large number of

PLL neurons were fluorescent (Fig. 1A). We documented the departures from the “normal” branching pattern at 1 mpf (Fig. 1A, arrows) and then cut the nerve in a very anterior position, so as to have an extended region in which to assess the faithfulness of path repeat. We observed that most variations were faithfully reproduced on regeneration (Fig. 1C and D, arrows) with a few changes (asterisks), mostly redundant branches that were not reestablished. Reinnervation took place progressively from proximal to distal (Fig. 1C).

Next we examined the dynamics of neuromast reinnervation throughout adulthood (1, 3, 6, and 15 mpf) by following the extent of neuromast reinnervation every day after a nerve cut. We observed that the speed of regeneration, represented as the slope of the reinnervation curve, did not change significantly between 1 and 6 mpf, with complete or almost complete regeneration within 5 d after the onset of reinnervation, but was significantly reduced in 15-mpf fish, in which only 25% of neuromasts were reinnervated during the same period (Fig. 2A; slope at 1 mpf, 20.3 ± 0.3 ; 3 mpf, 25.1 ± 1.8 ; 6 mpf, 19.7 ± 3.1 ; 15 mpf, 5.5 ± 2.1). In contrast, the latency between nerve transection and neuromast reinnervation increased steadily with fish age. This latency was quantified as the time at which reinnervation is 30% complete (*Materials and Methods*). The results show that the progressive increase in latency is statistically significant (1 mpf, 2.0 ± 0.3 ; 3 mpf, 4.8 ± 0.7 ; 6 mpf, 5.9 ± 0.8 ; 15 mpf, 10.8 ± 1.8 ; one-way ANOVA, $P < 0.001$; followed by Bonferroni's post hoc test: 1 mpf vs. 3, 6, and 15 mpf, $P < 0.001$; 3 vs. 6 mpf, $P < 0.05$; 3 vs. 15 mpf, $P < 0.001$; and 6 vs. 15 mpf, $P < 0.001$). In the following analyses, we concentrate on the factors that underlie this progressive increase in latency.

Precocious Regeneration After a Second Nerve Cut. We examined whether the nerve would regenerate again after a second cut, and if so, what would be the dynamics compared with the first

Fig. 2. PLL nerve regeneration in adult fish. (A) Extent of PLL nerve regeneration assessed as the proportion of reinnervated neuromasts (ordinate) over time (abscissa) in 1-, 3-, 6-, and 15-mpf fish. Values are means. (B–E) Delay in the onset of regeneration is reduced after a second cut at all ages (B, 1 mpf; C, 3 mpf; D, 6 mpf; and E, 15 mpf). NC1 is the first nerve cut, and NC2 is the second nerve cut. In B–D, comparisons always involved siblings. Differences in error bars between A and B–E reflect the fact that a small number of fishes died between the first and second nerve cuts.

regeneration process. We cut the PLL nerve for a second time, immediately posterior to the first cut, after complete reinnervation had been achieved (days 6, 7, 9, and 14 after the first nerve cut for 1-, 3-, 6-, and 15-mpf fish respectively; for 15-mpf fish, regeneration was partial even after 14 d). We observed that the nerve regenerates readily after the second cut and that the slope of the reinnervation curves is similar to the slope determined after the first cut at all ages (Fig. 2 *B–E*; slopes after second cut: 22.0 ± 1.2 at 1 mpf, 26.5 ± 1.9 at 3 mpf, 25.2 ± 1.9 at 6 mpf, and 6.1 ± 2.5 at 15 mpf). The latency of reinnervation, however, was dramatically reduced after the second nerve cut (Fig. 2 *B–E*; latencies after second cut: 1.5 ± 0.2 at 1 mpf, 3.0 ± 1.0 at 3 mpf, 3.4 ± 0.6 at 6 mpf, and 8.1 ± 0.9 at 15 mpf; Student *t* test, latencies after first vs. second cut: 1 mpf, $P < 0.01$; 3 mpf, $P < 0.001$; 6 mpf, $P < 0.001$; and 15 mpf, $P < 0.05$).

The observation that reinnervation is facilitated after a second cut suggests that some regeneration-promoting factor induced by the first cut is still present at the time of the second cut, thus reducing the delay before the onset of the second regeneration process. If this is the case, we would expect that longer time intervals between first and second nerve cuts would reduce the promoting effect. We measured the dynamics of reinnervation in 3-mpf fish on a second nerve cut done either 1 wk, as in the previous experiment (Fig. 2*C*), or 3 wk after the first nerve cut. The results show that whereas reinnervation takes place significantly earlier when the second cut is performed 1 wk after the first cut, this difference no longer exists when the second cut is performed 3 wk later (Fig. 3*A*; latency after first cut, 4.8 ± 0.7 ; after second cut 1 wk later, 3.0 ± 1.0 ; after second cut 3 wk later, 5.1 ± 0.7 ; one way ANOVA, $P < 0.001$, followed by Bonferroni's post hoc test: first cut vs. second cut 1 wk later, $P < 0.001$; first cut vs. second cut 3 wk later, nonsignificant; second cut 1 wk vs. 3 wk later, $P < 0.01$). We conclude that a previous nerve cut accelerates the first steps of axonal regeneration on a second cut but that this effect fades away over time and becomes negligible after 3 wk.

Fig. 3. Effect of time and position on a second regeneration. (A) The second cut was performed either 1 wk (NC2 1w, dashed line) or 3 wk (NC2 3w, dotted line) after the first cut (NC1, continuous line) in 3-mpf *nbt:dsred* adults. $P < 0.001$ for NC2 1w vs. NC1 and NC2 3w, one-way ANOVA followed by Bonferroni's post hoc test. (B) The second cut was performed either just posterior to the position of the first cut (NC2 post, dashed line) or 2–3 somites anterior to it (NC2 ant, dotted line). $P < 0.001$, NC2 post vs. NC1 and NC2 ant, one-way ANOVA followed by Bonferroni's post hoc test. (C) Regeneration at 1 mpf after nerve cut or after nerve ablation over two somites. (D) Promoting effect of a first cut when a second cut just distal to the lesion is accompanied by nerve ablation over the next two somites in 1-mpf fish. C and D were done on *nbt:dsred*; *Sox10-gfp* to verify the disappearance of Schwann cells. In each panel, the data were derived from sibling fishes.

We next examined whether the regeneration-promoting effect induced by a first cut involves global changes in the neurons or local changes at the level of the nerve cut (ie, changes in the axon or in the neighboring Schwann cells). If the promoting effect results from a global change in the neurons, cutting the nerve for a second time a few somites proximal to the position of the first cut would still result in an earlier onset of regeneration, as in the “conditioning effect,” in which a cut in the peripheral axon of dorsal root sensory neurons facilitates the regeneration of their central axon (12–14). In contrast, if local factors are involved in the regeneration-promoting effect, a second nerve cut proximal to the first one should not lead to accelerated reinnervation. This led us to perform a second cut of the nerve in 3-mpf fish 1 wk after the first nerve cut, either immediately posterior to the first cut, as in all previous experiments, or 2–3 somites anterior to it, and to assess the reinnervation dynamics afterward. We confirmed an earlier onset of reinnervation when the second cut is done just posterior to the first cut (Figs. 2*C* and 3*B*, dashed line). This regeneration-promoting effect is no longer evident, however, when the second cut is performed 2–3 somites anterior to the first one (Fig. 3*B*, dotted line; latency after the first cut, 5.9 ± 0.8 ; after a second cut just posterior to the first one, 3.4 ± 0.6 ; and after a second cut just anterior to the first one, 5.6 ± 0.7 ; one-way ANOVA, $P < 0.001$; followed by Bonferroni's post hoc test: latency of the first vs. a posterior second cut, $P < 0.001$; first vs. 2–3 somites anterior second cut, not significant).

We verified that the regeneration-promoting effect is not a peculiarity of the *nbt:dsred* line by performing the same experiment in another reporter line, *Hgn39d*, in which only lateral line neurons express GFP. We observed that in 1-mpf fishes, 30% of neuromast reinnervation is achieved 3.0 ± 0.5 d after a first cut. This value was significantly different when a second cut was performed immediately posterior to the first one (1.6 ± 0.4 d), but not when the second cut was performed two somites anterior to the first cut (2.7 ± 0.4 d; one way ANOVA, $P < 0.001$; followed by Bonferroni's post hoc test; latency after posterior second vs. first cut, $P < 0.01$; after anterior second vs. first cut, not significant). We conclude that both the regeneration-promoting effect and the fact that it acts locally are independent of the reporter line used.

Regenerating Axons Grow Along Schwann Cells but Do Not Need Them. The promoting effect observed when a second cut is done just distal rather than proximal to the first cut may be a result of several reasons, the most obvious of which are either an increased attractiveness of the distal Schwann cells, which have become deprived of axons after the first cut, or a local change in axonal properties. To discriminate between these two possibilities, we examined whether Schwann cells play any role during axonal regeneration.

Schwann cells surround the PLL nerve (15) and are responsible for the phagocytosis of axon debris during Wallerian degeneration in different neuronal populations (16). We first examined the fate of the Schwann cells surrounding the nerve distal to the cut, using a *Sox10-GFP* reporter line (17) in which *Sox10*-positive Schwann cells express GFP throughout adulthood. Schwann cells distal to the cut were not notably affected after axonal Wallerian degeneration had occurred (Fig. 4*A* and Fig. S2*A*). The regenerating axons grow closely apposed to Schwann cells in all cases (Fig. 4*B* and Fig. S2*B*), consistent with our previous observation that regeneration usually reproduces the detailed pattern of nerve branching (Fig. 1).

We then tested whether the presence of Schwann cells distal to the cut is required for nerve regeneration and/or guidance. To this end, the nerve cut was accompanied by complete nerve ablation through laser exposure, over one somite distal to the nerve cut, in *nbt:dsred* fishes. We observed that regeneration took place despite this treatment. It might have been, however, that

Fig. 4. Involvement of Schwann cells in axonal regeneration. (A) Three days after nerve cut in 1-mpf fishes, the axons have fully degenerated (double-headed arrow), but Schwann cells (arrowheads) are not noticeably affected posterior to the leading growth cones (arrows). (B) During regeneration, the leading growth cone (arrows) and neurite (double arrow) are closely apposed to Schwann cells (arrowheads). (C) 2 d after nerve ablation, Schwann cells are still absent from the exposed region (double-headed arrow), yet the growth cones of regenerating neurites (arrowheads) have crossed the glial gap. Regenerating neurites are notably defasciculated over the ablated region (arrows). (Scale bars, 50 μ m for A and B; 100 μ m for C.)

Schwann cells quickly reestablish a bridge over the ablated somite. We thus repeated the experiment in *nbt-dsred; Sox10-gfp* fishes. The result (Fig. 4C and Fig. S2C) confirms that nerve ablation effectively removes the Schwann cells over the ablated region and demonstrates that regenerating axons readily cross the Schwann cells gap. There is substantial defasciculation over the ablated region, however (Fig. 4C, arrows). Regenerating axons rerouted toward the original path along more posterior Schwann cells, and growth cones remained apposed to the Schwann cell tract distal to the ablated region (Fig. 4C, arrowheads). This result indicates that Schwann cells provide a preferred substrate for axon reelongation and act as a guidance cue for the regenerating nerve to advance along its former path, but they are not strictly required for regeneration to proceed.

Schwann cells, in turn, migrate along the newly established path to repopulate the ablated stretch during the next few days (Fig. 5A), showing that the regenerated nerve can act as a guidance cue for Schwann cells, as also happens during embryogenesis (18). Despite this reciprocal guidance effect, our results show that the presence of Schwann cells is not strictly required for regeneration itself.

Schwann Cells Are Not Involved in the “Promoting” Effect. Having established that Schwann cells are not required for nerve regeneration, we examined whether they play a role in the reduction of latency when axons regenerate after a second nerve cut. Because this effect could act over a distance of several hundred microns through diffusible growth factors, we first verified that axons still regenerate after nerve ablation over 2–3 somites ($>500 \mu$ m). They do, although they show extensive defasciculation and follow unexpected paths to cross the gap (Fig. 5B and C). In the absence of Schwann cells along the original nerve path, there is a clear tendency of regenerating neurites to follow Schwann cells along the nerve branches that lead to the neuromasts, as well as to follow the interneuromast cells that connect consecutive neuromasts (Fig. 5B, arrowheads), as already observed during embryogenesis (11). Axons may extend either rostrally or caudally (Fig. S2D) and may also grow back to the

lateral nerve along nerve branches (Fig. S2E”, arrows). Some regenerating stretches do not seem to correspond to any recognizable guiding cue, however (Fig. 5C, arrowheads).

Although regeneration does take place on Schwann cell ablation and leads to essentially complete reinnervation, we wondered whether it might be at all delayed. We first compared the dynamics of reinnervation in 1-mpf juveniles after a single cut, with or without Schwann cell ablation, over 2–3 somites. The results (Fig. 3C) show that there is a delay in reinnervation after Schwann cell ablation relative to nerve cut (latency after nerve cut, 2.2 ± 0.2 ; latency after nerve ablation over two somites, 3.0 ± 0.3 ; Student *t* test, $P < 0.001$), suggesting that the presence of distal Schwann cells facilitates regeneration. Next, to determine whether the distal Schwann cells play any role in the promoting effect of a first cut, we examined whether the regeneration-promoting effect is still observed if Schwann cells are ablated over 2–3 somites distal to the first cut. The rationale of the experiment was that if Schwann cells distal to the cut are involved in this effect, then ablating them should remove the effect. If, in contrast, the promoting effect is intrinsic to the axons, then it should still be observed when the distal Schwann cells are removed. The results (Fig. 3D; latency after nerve ablation, 2.23 ± 0.47 ; latency after nerve ablation after an earlier nerve cut, 1.59 ± 0.28 ; Mann–Whitney nonparametric test, $P < 0.001$) show that the promoting effect of a nerve cut is maintained even if the second cut is accompanied by nerve ablation over several somites posterior to the first cut. This result shows that the promoting effect does not depend on the presence of Schwann cells distal to the first cut and is, therefore, most likely intrinsic to the axons.

Discussion

In this work we examined whether axonal regeneration occurs in the PLL of adult and aging zebrafish and which rules it follows. We found that nerve regeneration and target organ reinnervation occur at all ages tested, from 1 to 15 mpf. Reinnervation was easily and reproducibly quantified, making the PLL a potentially useful system to study the process of axonal regeneration after an injury.

Two aspects of reinnervation turn out to be affected independently as animals age, suggesting the existence of two major components differentially affected by aging within the regeneration process. The first aspect is the rate of regeneration,

Fig. 5. Regeneration after extended nerve ablation. (A) In *nbt-dsred; Sox10-gfp* fishes, Schwann cells (arrowheads) extend over the ablated region (double-headed arrow) along the massively defasciculated regenerated axons. (B and C) Nerve ablation over 2–3 somites (double-headed arrows) does not prevent axonal regeneration but results in many axons following alternative courses over the ablated stretches (arrowheads). (Scale bars, 200 μ m.)

as represented by the slope of the reinnervation curve. This rate remains constant between 1 and 6 mpf but is markedly decreased at 15 mpf, evidencing a threshold age beyond which the regeneration process slows down. The second aspect is the latency before reinnervation takes place, which increases steadily with age from 1 to 15 mpf. We found that this latency is reduced when a second nerve cut is made distal to the first one. The rate of reinnervation, however, was not affected by this treatment. This differential response supports our assumption that the latency and the rate of reinnervation are components of the regeneration process that are differently affected by age, such that only the “latency” component increases progressively with age and is reduced (at all ages) when a second cut is done a few days after a first lesion.

The promoting effect of a first lesion has been demonstrated in the case of the “conditioning lesion” of dorsal root neurons, in which regeneration of the central axon is facilitated after the peripheral axon has been severed (12, 19). Unlike the conditioning effect, however, the promoting effect we document here is localized at, or distal to, the site of the first nerve cut. Although Schwann cells play a major role in the guidance of regenerating axons, as evidenced by the close apposition of the latter along the former, we observed that the absence of Schwann cells has but little effect on the latency of reinnervation. This is consistent with a similar observation made during early larval life (10). Thus, the promoting effect is likely to involve a local change in axonal properties, rather than an increased attractiveness of denervated Schwann cells.

The first steps of axonal regeneration after injury involve repairing the ruptured membrane with a sealing patch and assembling a new growth cone, a process that needs *de novo* protein synthesis (20). The shortened latency observed after a second cut distal to the site of the first lesion is consistent with the idea that essential components of the local machinery involved in growth cone assembly and neurite growth remain in place, either in the form of proteins or of locally stored mRNA (21), thus facilitating the development of a new growth cone after a second lesion. Promising candidates for these promoting factors are genes involved in axonogenesis and microtubule assembly or stabilization, such as Tau or GAP-43.

An important aspect of reinnervation that we have not addressed in this work is whether regenerating axons retain their original specificity in terms of somatotopy and of polarity selectivity. Both somatotopy and polarity selectivity have only been demonstrated for embryonic and early larval neurons (22–24), and whether either or both of these two features are also present in later developing axons, and maintained on regeneration, remains an open question.

Materials and Methods

Fish Care. Zebrafish (*Danio rerio*) were kept under standard conditions (25). The experimental animals' care was in accordance with the guidelines of University Montpellier 2. Ages are expressed as days postfertilization or mpf. The ages chosen for nerve regeneration analyses represent the juvenile stage (1–6 mpf), adulthood (3 and 6 mpf), and aged adults (15 mpf).

Nerve Cuts and Nerve Ablations. For 1–2-mpf juveniles, the PLL nerve was cut with a Micropoint laser system (Photonic Instruments) used on a Zeiss Axioplan 2 microscope equipped with a 40× water immersion objective,

using coumarin 440 nm, 5 mM in methanol, as a laser medium. Nerve ablation over several somites was performed in the same way. For 3-, 6-, and 15-mpf adults, a superficial incision of the skin was done with a broken microcapillary used as glass scalpel under a fluorescence dissection microscope (Olympus), and the nerve was subsequently cut with the same tool. After the cut was done, the head was freed from agar, and water was gently blown in the mouth for up to several minutes to help breathing, as described later. Nerve cuts were performed at the level of the anus, approximately at the 18th somite, except when otherwise specified.

In Vivo Imaging of Regenerating PLL Axons. Zebrafish juvenile and adult individuals were anesthetized with Tricaine (3-amino benzoic acid ethylester, Sigma A-5040, 4 mg/mL in 20 mM Tris at pH 7, diluted 25× in tank water just before use) and mounted on depression slides with 0.5% (juveniles) or 1.5% (adults) agar in tank water. Imaging was done either on a Leica SPE confocal microscope or on a Zeiss Axioimager equipped with a Coolsnap camera. Sensory neurons including their neurites were visualized in *nbt:dsred* fish, in which all neurons show red fluorescence (26) (kindly provided by M. van Drenth and D. Gilmour, Max Planck Institute for Developmental Biology, Tübingen, Germany), or in the *Hgn39d* line (27), kindly provided by H. Lopez-Schier (Centre for Genomic Regulation, Barcelona). Schwann cells were visualized in *sox10:GFP* fish (17), kindly provided by Robert Kelsh (University of Bath, Bath, UK). Neuromasts were visualized either in the *SqET20* line (28) (kindly provided by M. Allende, Universidad de Chile, Santiago, Chile), where neuromast mantle cells and interneuromast cells show green fluorescence, or by incubating fish for 5 min in 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DiAsp 2 μg/mL; Sigma), a vital dye taken up by hair cells, previous to anesthetizing. Imaging sessions of adult fish to assess ongoing PLL nerve regeneration lasted 10 min or less, a period during which anesthesia is well tolerated by adults. It was found necessary, however, to gently blow tank water through their mouth with a P200 pipette for up to several minutes at the end of the imaging session, to help breathing and allow full recovery.

Quantification of Nerve Regeneration. Wallerian nerve degeneration was revealed by a punctate labeling distal to the lesion and disappearance of the branches that innervate the neuromasts. Nerve regeneration was assessed daily after the nerve cut by examining the proportion of stitches that had become reinnervated. We quantified reinnervation using two parameters: the slope, which represents the rate of reinnervation, and the temporal delay between nerve cut and the time when 30% reinnervation is achieved, which we called latency. We chose this because, as we rely on the extent of reinnervation to assess nerve regeneration, the exact onset of regeneration cannot be accurately determined.

Blastoderm Cell Injection. Approximately 40–60 cells were drawn from a *nbt:dsred* donor embryo at 3.5–4 h, before the onset of epiboly, and immediately introduced into a *SqET20* receptor embryo of similar age. Cells were taken with a capillary needle, 20-μm tip diameter, held on a Narishige micromanipulator and connected to a Hamilton manual injector, under a dissection microscope. Grafted embryos were transferred to glass Petri dishes and kept at 29 °C for 48 h in 0.5 mg/L methylene blue in tank water. They were then raised in standard conditions.

Statistical Analysis. Comparisons were performed using Student *t* test (two tailed) or Mann–Whitney for pair comparisons and one-way ANOVA for multiple comparisons. ANOVA was followed by the post hoc Bonferroni's test to assess the *P* value of pair-comparisons within the multiple comparison. Differences were considered significant when *P* < 0.05. In all cases, raw data were previously tested for normality and homoscedasticity criteria.

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