Developmentally Regulated Impediments to Skin Reinnervation by Injured Peripheral Sensory Axon Terminals

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Summary

The structural plasticity of neurites in the central nervous system (CNS) diminishes dramatically after initial development, but the peripheral nervous system (PNS) retains substantial plasticity into adulthood. Nevertheless, functional reinnervation by injured peripheral sensory neurons is often incomplete [1–6]. To investigate the developmental control of skin reinnervation, we imaged the regeneration of trigeminal sensory axon terminals in live zebrafish larvae following laser axotomy. When axons were injured during early stages of outgrowth, regenerating and uninjured axons grew into denervated skin and competed with one another for territory. At later stages, after the establishment of peripheral arbor territories, the ability of uninjured neighbors to sprout diminished severely, and although injured axons reinitiated growth, they were repelled by denervated skin. Regenerating axons were repelled specifically by their former territories, suggesting that local inhibitory factors persist in these regions. Antagonizing the function of several members of the Nogo receptor (NgR)/RhoA pathway improved the capacity of injured axons to grow into denervated skin. Thus, as in the CNS, impediments to reinnervation in the PNS arise after initial establishment of axon arbor structure.

Results

Regenerating Sensory Axons Avoid Their Former Territories in the Skin at Later Developmental Stages

The peripheral axons of trigeminal sensory neurons terminate in elaborate arborizations within the skin of the head to mediate somatosensation. Although it is well known that the primary axons of peripheral sensory neurons possess substantial regenerative capacity (reviewed in [1]), few studies have examined the regenerative potential of unmyelinated axon terminals in the skin. We used zebrafish trigeminal neurons as a model to investigate the developmental regulation of cutaneous axon terminal plasticity and whether deficits in skin reinnervation may contribute to incomplete recovery following injury.

The skin of larval zebrafish consists of two epithelial layers, between which trigeminal axon terminals reside. These axons repel one another as they arborize to partition epidermal territory, leading to complete, minimally redundant innervation of the head by 36 hr postfertilization (hpf) [7]. To study the developmental control of skin reinnervation after injury, we severed the peripheral arbor of zebrafish trigeminal neurons expressing green fluorescent protein (GFP) with a femtosecond laser (see Figure S1 available online) [8], imaged their regeneration in live embryos for 12 hr, and traced their structure to quantify the success of target reinnervation (Figure 1; Movies S1–S13).

To specifically study axon regeneration within the skin, we directed all axotomies to second- or third-order axon branches, which are situated between the two skin layers. Axon regeneration was analyzed at three stages: 30 hpf, when trigeminal peripheral axons are still actively arborizing; 54 hpf, ~18 hr after arborization is complete; and 78 hpf. In all cases, the distal portions of severed axons degenerated and were cleared within 3 hr, leaving a denervated region of skin. Regenerating axons always partially reinnervated their former territories after axotomy at 30 hpf, but their capacity for reinnervation was significantly diminished after axotomy at 54 hpf and virtually nonexistent at 78 hpf. The portion of former territory reinnervated decreased from 47.5% ± 7.9% at 30 hpf to 0.3% ± 0.2% at 78 hpf (p < 0.0001); the percent of new growth that entered the denervated region decreased from 58% ± 6.3% at 30 hpf to 12.4% ± 8% at 78 hpf (p = 0.0002; Figure 2; Table S1). Most axons severed at 78 hpf initiated new growth but actively avoided their former territories (Figures 2G–2I). Avoidance was manifested as several distinct axon behaviors (Figure S2), including stalling, skirting the edge of the territory, or turning sharply, none of which was observed after axotomy at 30 hpf. Strikingly, although one-third (6 of 18) of axons severed at 78 hpf reentered their former territory, reinnervation was always transient: axons that entered the denervated territory either retracted (2 of 6; Figures S2F–S2J) or degenerated locally (2 of 6), or the parent neuron died (2 of 6; Figures S2K–S2O). Neither phagocytic blood cells nor peripheral myelin was responsible for inhibition, because avoidance was still observed in cloche mutant fish, which lack all blood cells [9], and in fish treated with the drug AG1478, which lack peripheral myelination (Figure S3 and Table S2) [10]. Axon avoidance of former territory was a persistent effect in that axons imaged for 5 days after 78 hpf axotomy never reinnervated their former territory (n = 7; Figure S4).

Developmental Regulation of Axon Plasticity and Tiling

During early larval zebrafish development, trigeminal axons repel one another to partition epidermal territory [7]. When arbor were removed early in development, neighboring axons expand to fill denervated territory. This strategy for partitioning arbor territories, known as “tiling,” is often employed when multiple neurons of the same type innervate a two-dimensional target. Although the arbors of some tiled neurons retain plasticity into adulthood (e.g., [11, 12]), allowing them to compensate for the loss of neighbors and to create new

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innervation patterns after injury, many populations of tiled neurites cannot reorganize after developmental critical periods (e.g., [13–15]).

To characterize the territory-partitioning strategy of trigeminal sensory terminals after injury at different developmental stages, we first investigated the ability of uninjured axons to sprout into newly denervated territory. To accomplish this, we laser ablated every cell within a trigeminal ganglion (Figures S1E and S1F) and calculated the axon growth rate from the uninjured contralateral ganglion into newly denervated territory. When a trigeminal ganglion was ablated at 30 hpf, uninjured axons from the contralateral ganglion reinnervated most of the denervated skin within 12 hr (Figure 3A). Following ablation at 78 hpf, growth into denervated territory was markedly reduced, and very little branching occurred (Figures 3B and 3C; Table S3; mm/hr/tip: 30 hpf 5.7 ± 0.3, 78 hpf 0.5 ± 0.2; p = 0.0071). Thus, there was a critical period after which the growth of uninjured axons into denervated territory diminished severely.

To test directly the hypothesis that uninjured and regenerating axons compete for denervated territory after injury at 30 hpf, we transplanted wild-type cells into neurogenin-1 morphants, which lack somatosensory neurons, generating zebrafish with a single trigeminal neuron [7]. Strikingly, isolated axons severed at 30 hpf reinnervated virtually all (98.1% ± 1.9%) of their former territories and grew beyond them (Figures 3E and 3H). In contrast, control axons (wild-type cells transplanted into wild-type embryos) only partially (27.3% ± 16.1%; p = 0.008) reinnervated their former territories (Figures 3D and 3H). Thus, incomplete reinnervation of former territory at 30 hpf was not due to a nonpermissive environment in the denervated region but rather to competition with uninjured neighboring axons (Figure 3I).

The peripheral axons of isolated neurons grow until they fill the entire head, long past 78 hpf [7]. However, following axotomy of isolated arbors at 78 hpf, regenerating axons avoided their former territories, with no significant change in the percent area reinnervated compared to wild-type control transplants (1.04% ± 0.8% versus 5.4% ± 4.3%; p = 0.3387; Figures 3F–3H). These results demonstrate that the denervated region actively repels regenerating axons at older developmental stages and that neither diminished growth rate nor competition from uninjured neighboring axons explains the lack of reinnervation (Figure 3I). Thus, contrary to expectation, the peripheral nervous system (PNS) is not always a permissive environment for regeneration: at later larval stages, local factors persistently marking former territories repel regenerating axons.

A Nogo Receptor/RhoA Pathway Is Required for Inhibition of Skin Reinnervation

We hypothesized that axons in the central nervous system (CNS) and PNS use similar molecular mechanisms to respond
to inhibitors in their respective environments. In the CNS, myelin-associated inhibitors activate a receptor complex that includes Nogo receptor (NgR) and LINGO-1 to block axon regeneration [16–19]. This complex activates an intracellular signaling cascade that involves the small GTPase RhoA, Rho kinase (ROCK), and collapsin response mediator protein-2 (CRMP-2) to cause growth-cone collapse [20, 21]. Zebrafish trigeminal neurons express homologs of NgR (ZF NgR), LINGO-1 (LINGO-1a), and CRMP-2 during larval stages [22–26].

To test whether the NgR pathway functions in peripheral sensory axon regeneration, we misexpressed dominant-negative (DN) versions of ZF NgR, LINGO-1a, and CRMP-2, as well as human RhoA, in trigeminal neurons, along with GFP [27–30]. Expression of all of the DN transgenes increased the fraction of regenerating axons that entered their former territories at 78 hpf compared to control axons coexpressing GFP and RFP or axons expressing full-length genes (Figure 4; Figure S5 and Table S2). As an additional control, we mutated a conserved amino acid in the DN NgR transgene (D163A) required for NgR binding to LINGO-1 and all known NgR ligands [31]. Axons expressing DN NgR D163A avoided their former territory, similar to controls and significantly different from those expressing DN NgR (Figures 4B and 4D; Figures SSD and S5K; Table S2). Blocking ROCK with the specific inhibitor Y-27632 [32] also improved reinnervation (Figure S6 and Table S2).

To rule out nonspecific effects of DN transgenes, we further investigated the function of ZF NgR, LINGO-1a, and CRMP-2 with morpholino antisense oligonucleotides (MO) targeting those genes. In all three cases, regenerating axons grew into denervated territory better than axons injected with a control MO (Figure 4; Figures SSG–SSJ and Table S2). Expression of the full-length LINGO-1a cDNA in neurons rescued the defect in repulsion observed in LINGO-1a morphants (Figure 4; Table S3). Blocking ROCK with Y-27632 also improved axon growth into denervated territory (Figure S6).

To determine when this pathway is required to inhibit outgrowth of trigeminal axons in the skin, we injected Y-27632 into embryos 2 hr before axotomy, as well as 1, 4, or 12 hr after axotomy (Figure S6 and Table S2). Reinnervation was only improved when ROCK was inhibited ≤4 hr after axotomy. To determine whether the same pathway was responsible for diminishing the ability of intact neighboring axons to sprout at 78 hpf, we injected Y-27632 2 hr before ablation of an entire trigeminal ganglion. ROCK inhibition had no effect on the ability of uninjured axons from the intact ganglion to sprout across the midline (Table S3). Similarly, intact axons in LINGO-1a morphants did not grow into denervated territory after we ablated a ganglion (Table S3). These results indicate that distinct pathways regulate the ability of injured and intact axon arbors to grow into denervated territory.

Discussion

It is generally believed that, in contrast to the CNS, the periphery is permissive for axon regeneration. Although substantial functional recovery from PNS injury can occur in adulthood, it is often incomplete (reviewed in [2]). Two phenomena are known to contribute to suboptimal PNS axon regeneration: the degeneration of supportive conduits that guide axons to the periphery and the inability of regenerating sensory endings to penetrate the epidermis [1–6]. We show here that inhibitory regions of skin can also be an obstacle to peripheral reinnervation. After a developmental critical period, regenerating cutaneous sensory axon endings specifically avoid reinnervating their former territories. There is a crucial distinction between regeneration and reinnervation: severing an axon stimulates its growth, but factors in the original territory prevent reinnervation. Interestingly, severing axons in the CNS also stimulates exploratory activity,
but very little reinnervation occurs [33], perhaps because axons are repelled by inhibitors expressed by myelin. When a regenerating trigeminal peripheral axon does grow into its former territory, it inevitably retracts, or the cell dies, suggesting that repellents in this skin region may also be toxic to neurons. These local impediments to full reinnervation of the skin may contribute to deficits in functional recovery from peripheral injury [2].

Because inhibition is limited to a region around a degenerated axon’s original territory, we speculate that axons alter the extracellular matrix (ECM), leaving behind a persistent “ghost” that demarcates their former territories. This local mark of an axon’s territory might be laid down at a specific developmental stage to stabilize arbors. One possibility is that inhibitory factors derive from membrane-associated environments. It is possible that known ligands of the NgR complex, expressed in a different context, inhibit peripheral regeneration, but because these proteins are structurally diverse, it is also possible that different ligands functions in the periphery.

Our studies reveal a critical period for the plasticity of somatosensory arbors that limits their ability to respond to injury and fully reinnervate the skin. We propose that after initial development, local inhibitors stabilize sensory axons in the skin, consequently limiting their ability to reinnervate it after injury. Several molecular studies link functional recovery and axon regeneration following spinal cord injury to developmental plasticity in the CNS [34–38]. The NgR pathway limits ocular dominance plasticity in the visual cortex [34], in addition to limiting regeneration in response to CNS injury [39, 40] and, as we have now shown, PNS injury. Our results support the idea that impediments to recovery from injury to the mature CNS axons are a consequence of the stabilization of neuronal structure that occurs when initial morphogenesis ends.

**Experimental Procedures**

As previously described, a GFP reporter transgene was used to visualize trigeminal neurons (Tg(sensory:GFP)) [7], as well as a variation expressing GFP and dominant-negative (DN) NgR (B), or DN RhoA (C) is shown. Data to the left of the double bar are from axons expressing GFP and dominant-negative or full-length versions of the genes indicated. Data to the right of the double bar are from Tg(sensory:GFP) embryos injected with indicated morpholinos. Red indicates fraction of axons that grew into denervated territory; blue indicates axons that avoided denervated territory (see Supplemental Experimental Procedures). See Table S2 and Movies S11–S13.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures, three tables, six figures, and thirteen movies and can be found online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01913-7.

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**References**

1. Chen, Z.L., Yu, W.M., and Strickland, S. (2007). Peripheral regeneration. Annu. Rev. Neurosci. 30, 209–233.
2. Hoke, A. (2006). Mechanisms of disease: What factors limit the success of peripheral nerve regeneration in humans? Nat. Clin. Pract. Neurol. 2, 448–454.
3. Navarro, X., Verdu, E., Wendelschafer-Crabb, G., and Kennedy, W.R. (1997). Immunohistochemical study of skin reinnervation by regenerative axons. J. Comp. Neurol. 380, 164–174.
4. Rajan, B., Polydefkis, M., Hauer, P., Griffin, J.W., and McArthur, J.C. (2003). Epidermal reinnervation after intracutaneous axotomy in man. J. Comp. Neurol. 457, 24–36.
5. Speidel, C.C. (1964). In vivo studies of myelinated nerve fibers. Int. Rev. Cytol. 16, 187–231.
6. Verdes, E., and Navarro, X. (1997). Comparison of immunohistochemical and functional reinnervation of skin and muscle after peripheral nerve injury. Exp. Neurol. 146, 187–198.
7. Sagasti, A., Guido, M.R., Raible, D.W., and Schier, A.F. (2005). Repulsive interactions shape the morphologies and functional arrangement of zebrafish peripheral sensory arbors. Curr. Biol. 15, 804–814.
8. O’Brien, G.S., Pieper, S., Martin, S.M., Cavanaugh, A.M., Portera-Cailliau, C., and Sagasti, A. (2009). Two-photon anatomy and time-lapse confocal imaging in live zebrafish embryos. J. Vis. Exp. Published online February 16, 2009. 10.3791/1129.
9. Stainier, D.Y., Weinstein, B.M., Detrich, H.W., 3rd, Zon, L.I., and Fishman, M.C. (1995). Croche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development 121, 3141–3150.
10. Lyons, D.A., Pogoda, H.M., Voas, M.G., Woods, I.G., Diamond, B., Nix, R., Arana, N., Jacobs, J., and Talbot, W.S. (2005). erbB3 and erbB2 are essential for schwann cell migration and myelination in zebrafish. Curr. Biol. 15, 513–524.
11. Blackshaw, S.E., Nicholls, J.G., and Parnas, I. (1982). Expanded receptive fields of cutaneou N reproduced receptors after single neuron deletion in leech central nervous system. J. Physiol. 326, 261–268.
12. Scott, S.A., Macintyre, L., and Diamond, J. (1981). Competitive reinnervation of salmonader skin by regenerating and intact mechanosensory nerves. Proc. R. Soc. Lond. B. Biol. Sci. 211, 501–511.
13. Eysel, U.T., Peichl, L., and Wassle, H. (1985). Dendritic plasticity in the early postnatal feline retina: Quantitative characteristics and sensitive period. J. Comp. Neurol. 242, 134–145.
14. Jackson, P.C., and Diamond, J. (1981). Regenerating axons reclaim sensory targets from collateral nerve sprouts. Science 214, 926–928.
15. Sugimura, K., Yamamoto, M., Niwa, R., Satoh, H., Goto, S., Taniguchi, M., Hayashi, S., and Uemura, T. (2003). Distinct developmental modes and lesion-induced reactions of dendrites of two classes of Drosophila sensory neurons. J. Neurosci. 23, 3752–3760.
16. Filbin, M.T. (2003). Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat. Rev. Neurosci. 4, 703–713.
17. Fournier, A.E., GrandPre, T., and Strittmatter, S.M. (2001). Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409, 341–346.
18. Liu, B.P., Fournier, A., GrandPre, T., and Strittmatter, S.M. (2002). Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. Science 297, 1190–1193.
19. Wang, K.G., Kopricua, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., and He, Z. (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417, 941–944.
20. Mimura, F., Yagamiushi, S., Arimura, N., Fujitani, M., Kubo, T., Kaibuchi, K., and Yamashita, T. (2003). Myelin-associated glycoprotein inhibits microtubule assembly by a Rho-kinase-dependent mechanism. J. Biol. Chem. 278, 1970–1979.
21. Niederost, B., Oertle, T., Fritsche, J., McKinney, R.A., and Bandtlow, E.C. (2002). Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J. Neurosci. 22, 10368–10376.
22. Brosamle, C., and Halpern, M.E. (2008). Nogo–Nogo receptor signalling in PNS axon outgrowth and pathfinding. Mol. Cell. Neurosci. 40, 401–409.
23. Christie, T.L., Starovic-Subota, O., and Childs, S. (2006). Zebrafish collapsin response mediator protein (CRMP)-2 is expressed in developing neurons. Gene Expr. Patterns 6, 193–200.
24. Klinger, M., Taylor, J.S., Oertle, T., Schwab, M.E., Stuermier, C.A., and Diekmann, H. (2004). Identification of Nogo-66 receptor (NgR) and homologous genes in fish. Mol. Biol. Evol. 21, 76–85.
25. Schick, B., Becker, C.G., Schachner, M., and Becker, T. (2005). Expression of collapsin response mediator proteins in the nervous system of embryonic zebrafish. Gene Expr. Patterns 5, 809–816.
26. Thibes, B., Wright, G.J., and Thibes, C. (2008). Embryonic and larval expression patterns from a large scale screening for novel low affinity extracellular protein interactions. ZFIN Direct Data Submission, http://zfian.org/cgi-bin/webdriver ~Mvial=aa-pubview2. apg&OID=ZDB-PUB-080227-22.
27. Domeniconi, C., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K., Nukilina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., et al. (2002). Myelin-