Pre-existing pathways promote precise projection patterns

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A large body of evidence shows that molecular cues promote specific synapse formation by guiding axons and by mediating their association with targets, but much less is known about the contribution of physical cues (such as mechanical constraints) to these processes. Here we used the peripheral motor system to investigate the latter issue. In living mice, we viewed individual motor axons bearing a fluorescent reporter, and mapped the cohort of muscle fibers that they innervated both before and after nerve damage. When gross trauma was minimized (by a nerve-crushing rather than nerve-cutting procedure), regenerating axons retraced their former pathways, bifurcated at original branch points, and formed neuromuscular junctions on the same fibers that they originally innervated. Axonal growth through tubes of non-neural cells seemed to account for this specificity, and specificity degraded when the tubes were cut. These results suggest that nonspecific guidance cues can be sufficient to generate specific synaptic circuitry.

In mammals, axons of the peripheral and central nervous systems respond differently to damage: central axons are strongly inhibited from regenerating, whereas peripheral axons are capable of growing long distances and reestablishing functional connections. This functional regeneration has been shown for motor neurons in muscles, sensory neurons in sensory end organs, and autonomic preganglionic and postganglionic cells in autonomic end organs[1–10]. In some cases, functional recovery after such injury is good[5–6], but in other cases, even when regeneration itself is robust, the axons can synapse on inappropriate targets. When motor axons reinnervate 'incorrect' muscles, it results in uncoordinated movement; misrouted regenerating sensory axons cause sensory disturbances; and imprecise connectivity in the autonomic nervous system leads to disruption of autonomic reflexes[2,7–9]. The quality of reinnervation may be related to whether or not the nerve trunk was severed at the injury site and the extent to which Schwann cell tubes within the peripheral nerve stump remain intact[10]; once in the distal stump, axons grow preferentially through such trunks and tubes.

Knowing the degree to which synaptogenesis is specific after peripheral injury has three main implications. First, this knowledge will enable more accurate predictions of central reinnervation if current efforts to overcome barriers to axonal regeneration prove successful. Second, noting the imperfections in the regeneration process will inform new methods for improving clinical outcomes. And finally, this work sheds light on the processes that establish specific connectivity during embryonic development. Unfortunately, however, assays of the quality of reinnervation have so far been imprecise. In the motor system, for example, it is possible to determine whether axons from appropriate populations (such as motor neurons from the appropriate rostrocaudal region of the spinal cord) re-connect with correct targets such as particular muscles[11–13] or segments of muscles[14–17], but it has not been possible to determine whether individual axons ever reinnervate their previous cellular targets. Here we analyzed reinnervation at the cellular level, using transgenic mice that express fluorescent proteins in their axons[18]. Our aim was to map the muscle fibers that single motor axons innervated both before and after reinnervation. We expected, after nerve crush, that labeled axons would enter the distal nerve stump and use this path as a guide back to the same muscle they had originally innervated. We found that axons actually re-entered the same tubes and reinnervated exactly the same muscle fibers that they had previously contacted. Indeed, this high quality of re-innervation suggests that many cases of axotomy may go entirely unnoticed and subclinical injury may be more common than it is presently thought to be.

Results

Reconstitution of motor units after nerve crush

To visualize motor axons and neuromuscular junctions in live animals, we used transgenic mice in which a yellow spectral variant (YFP) of the green fluorescent protein (GFP; ref. 19) was expressed in the cytoplasm of cells under the control of neuron-specific regulatory elements from the *thy1* gene[18]. All motor axons were labeled in most thy1-YFP mouse lines (**Fig. 1a**), but in a few, only a small and seemingly random subset of motor axons was labeled (**Fig. 1b**; ref. 20). Patterns of expression were heritable in each line and were hardly affected by genetic background[18]. For the studies reported here, we used one line, thy1-YFP-H, in which up to five motor axons were fluorescent in each of several skeletal muscles of the neck. Fluorescence extended throughout the cytoplasm of axons that expressed the transgene, completely labeling nerve terminals, each of which was apposed to an acetylcholine receptor (AChR)-rich postsynaptic apparatus at a neuromuscular junction (**Fig. 1c**).

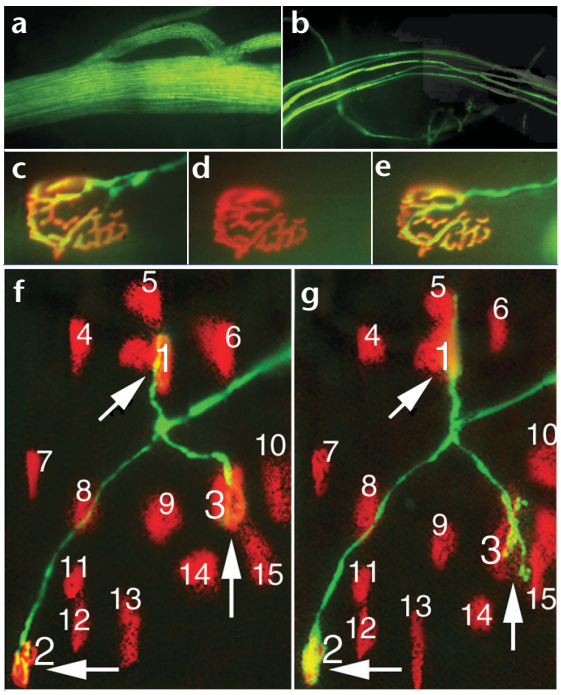

Fig. 1. Selective reinnervation of original synaptic sites on original target cells in the sternomastoid muscle. (**a**, **b**) Muscle nerves from thy1-YFP-F (**a**) and thy1-YFP-H (**b**) mice, showing that all axons are labeled in the former but only a subset in the latter. Because of this limited labeling, thy1-YFP-H mice were used in all subsequent experiments. (**c**–**e**) A rhodamine-α-bungarotoxin (rBTX)–labeled synaptic site (red) on a muscle fiber was identified before (**c**), two days after (**d**), and seven days after (**e**) nerve crush. (**e**) The YFP-labeled axon (green) degenerated after axotomy, then regenerated to precisely reoccupy its original synaptic site. (**f**, **g**) Field containing 15 (numbered) rBTX-labeled synaptic sites before (**f**) and 7 days after (**g**) axotomy. The labeled axon originally occupied 3 of the 15 sites (arrows); it reinnervated all 3 of these sites but no others (~20) in this field of view. This field was also imaged one day after axotomy to confirm that the axon had degenerated (data not shown).

We first examined YFP-labeled axons that innervated superficial (ventral) muscle fibers of the sternomastoid muscle, where re-imaging the same neuromuscular junctions in living mice over weeks or months is routine[21,22]. In these mice, branches of YFP-expressing motor axons innervated some superficial muscle fibers but not others. In each mouse, we crushed the motor nerve to the left sternomastoid at a single site proximal to its muscle entry: 5 mm to 1 cm from the superficial neuromuscular junctions[21]. This type of injury severs all the axons within the nerve, but leaves the perineurium surrounding the bundle of axons as well as the distal Schwann cell tubes intact[23–27]. We re-imaged the muscles two days later and, as expected, labeled axons had degenerated (**Fig. 1d**). The animals were returned to their cages and then, 7–28 days later, after the nerve had fully regenerated[9], the muscles were re-examined. In each reinnervated muscle, a labeled axon was visible. The YFP-labeled motor nerve terminals were again apposed to previously identified AChR-rich postsynaptic specializations (**Fig. 1e**), confirming the reinnervation of original synaptic sites documented previously in several vertebrate species, including mice[21,27].

What was unexpected was that in each case, the regenerated labeled motor axon made most of its new synapses on the same muscle fibers that it had innervated originally (**Fig. 1f** and **g**). Regenerated axons successfully re-innervated 90% of their original target muscle fibers, and 93% of their synapses were on these original target fibers (**Table 1**, lines 1–3; $n = 3$). This distribution was highly non-random as assessed by χ^2 test ($P < 0.001$), indicating a hitherto unsuspected precision in the specificity of muscle reinnervation.

Regrowth of axons along pre-existing pathways

Accurate reconstitution of original motor units could arise if regenerating axons (i) recognized their original target muscle fibers or (ii) were guided by pathways that lead to these targets. To distinguish between these alternatives, we used the platysma muscle of the neck instead of the sternomastoid because is only a few fibers thick, which permits its entire depth to be imaged *in vivo*. This favorable geometry allowed us to map nearly the entire intramuscular trajectory of a single motor axon and to identify the target muscle fibers in a motor unit (**Fig. 2**). After mapping a motor unit, the nerve fascicle containing the labeled axon (and neighboring unlabeled axons) was crushed several millimeters from its terminal field (red "X" in **Fig. 2a**). When muscles were re-imaged 25 days later, the orientation was sometimes altered, perhaps due to changes in the surgical exposure of the muscle. Nonetheless, the vast majority of neuromuscular junctions could be re-identified and, as in the sternomastoid, motor axons selectively reinnervated their original postsynaptic target cells (compare **Fig. 2a** and **c** with **Fig. 2b** and **d**; **Table 1**, axons 4–9). Notably, the montages generated from images obtained before nerve crush and after regeneration indicate that axons retraced their original pathways to reach these targets.

In normal muscles, each motor axon is surrounded by an endoneurial sheath consisting of a myelin-forming Schwann cell

Table 1. Reinnervation of original targets after nerve crush.

Axon	Original sites	Total new synapses	(% of original)	New synapses at original site	(% of all new)
1	3	3	(100)	3	(100)
2	14	9	(64)	8	(89)
3	2	2	(100)	2	(100)
4	7	6	(86)	6	(100)
5	8	4	(50)	4	(100)
6	26	16	(62)	16	(100)
7	19	13	(68)	12	(92)
8	12	9	(75)	7	(78)
9	26	26	(100)	26	(100)
10	30	21	(70)	21	(100)
11	8	2	(25)	2	(100)
12	10	7	(70)	7	(100)
Average	13.8 ± –2.8	9.8 ± –2.3	(72.5 ± 6.7)	9.5 ± 2.3	(96.6 ± –2.1)

Axons 1–3 were from thy1-YFP-H sternomastoid muscles; axons 4–9 were from thy1-YFP-H platysma muscles; axons 10–12 were YFP-positive axons from platysmas of thy1-YFP-H/thy1-CFP-D double transgenics. Reinnervation was assessed 7 (sternomastoid) or 25 days (platysma) after axotomy. Data was obtained from montages; data shown in **Fig. 1f** and **g**, **Fig. 2** and **Fig. 3a–d** correspond to axons 1, 9 and 10, respectively. Average, mean ± s.e.m.

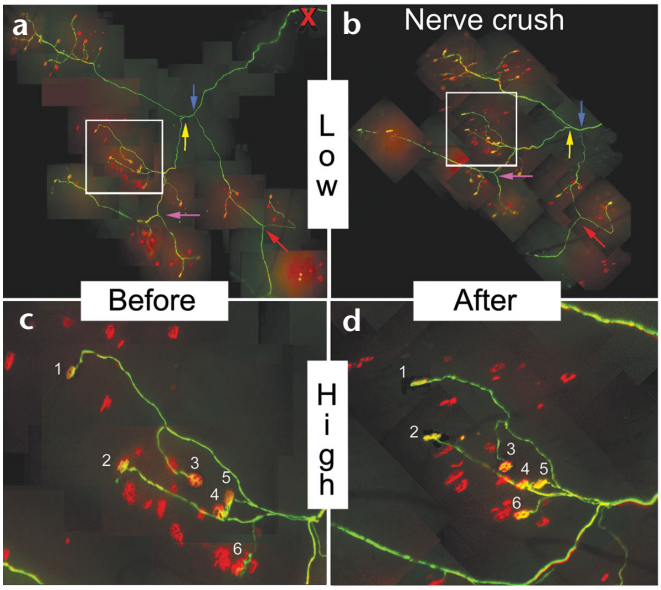

Fig. 2. Axons retrace original paths to reach original targets in the platysma muscle. A field approximately 5 × 5 mm was imaged before (**a**, **c**) and 25 days after nerve crush (**b**, **d**). (**a**, **b**) Colored arrows show corresponding branching points. (**a**) Red 'X' (top right) shows site of nerve crush. (**a**, **b**) Region boxed in (**c**, **d**) shown at higher magnification. The labeled axon reoccupied all six of the synaptic sites that it occupied originally and reached them by the original route.

and its basal lamina; fascicles of axon/Schwann cell units are wrapped by a perineurial sheath, consisting of specialized fibroblasts and their basal lamina[28]. Distal endoneurial and perineurial sheaths persist after axotomy, and axons preferentially regenerate through these sheaths when they reinnervate muscles after nerve crush[23–25,29,30]. The extent to which individual axons retrace their original routes, however, remains unknown. In the platysma, the former nerve trunks survived and, as expected, regenerating axons were largely restricted to them. Within these trunks, individual regenerating axons seemed to enter their original tubes—they precisely retraced their original paths, faithfully reestablishing branches at nearly every former branch point, including those several millimeters from their terminals (**Fig. 2**). On average, single YFP-labeled axons branched 15 times within their intramuscular course. After regeneration, axons established ~20% fewer branches, but ~95% of the bifurcations that did form were localized precisely to the sites at which the axon had bifurcated before axotomy (**Table 2**).

Although these results are consistent with the idea that each individual axon projects with little error to its previous targets, we considered the alternative possibility that axons might initially explore many nerve branches and then trim collaterals to generate a precise projection pattern. We imaged the pattern of reinnervation three days after nerve crush in the sternomastoid muscle in transgenic mice (YFP-F) in which YFP was expressed in all motor axons (six mice). At three days after nerve crush, approximately half of the neuromuscular junctions were completely reinnervated; the rest were either partially occupied or, in some cases, a growth cone could be seen approaching an unoccupied postsynaptic site. In no cases ($n > 100$ neuromuscular junctions) were multiple axons seen traversing the same paths or converging on the same junction. This suggests that there is little extra branching at the earliest stages of reinnervation. Given that at later stages of reinnervation there is only 10–20% extra branching[21] after a single nerve crush, at most, there seems to be little opportunity for large scale exploration and refinement to generate the precise axonal projections seen here. Although we cannot exclude the possibility that exploratory behavior of regenerating axons leads to some transient errors, our results suggest that, for the most part, axons only re-enter their former tubes and reestablish their former branching pattern in response to localized bifurcation-inducing signals that persist at previous branch points in the nerveless distal stump.

Table 2. Reconstitution of intramuscular branching pattern after nerve crush.

Axon	Original branches	New branches	(% of original)	New branches at original site	(% of all new)
4	11	9	(82)	9	(100)
5	9	7	(78)	7	(100)
6	20	14	(70)	14	(100)
7	18	10	(56)	9	(90)
8	10	8	(80)	6	(75)
9	21	21	(100)	21	(100)
10	5 [14][a]	3	(60)	3	(100)
11	22 [42]	13	(59)	10	(77)
12	25 [72]	20	(80)	20	(100)
Average	15.7 ± –2.5	11.7 ± –2.1	(73.9 ± 5.2)	11.0 ± –2.2	(93.6 ± –3.7)

Axon numbers correspond to those in **Table 1**.
[a]Numbers in brackets indicate total number of branch points that the YFP-labeled axon encountered in the area imaged, as revealed by courses of CFP-labeled axons in the same nerve trunk (**Fig. 3f** and **g**).

Reinnervation in doubly labeled transgenic mice

To better understand what accounted for the large number of correctly reinnervated targets as well as why there were occasional errors, we studied single axons that were labeled differently from a background of other axons. This way, we could determine whether the labeled axon was the only axon to take a particular path. In addition, we could learn whether the few branch points that were not taken and the synaptic targets that were not reoccupied by YFP-labeled axons had been taken and reoccupied by other axons. To generate these two-color (yellow and cyan variants of GFP) mice, we crossed thy1-YFP-H mice with thy1-CFP-D mice[18] to obtain double-transgenic progeny within which all motor axons were CFP-positive and a small subset (often one axon) was also YFP-positive. Selective excitation and emission filters enabled us to visualize each fluorophore separately and to distinguish the complete arbor of one YFP-labeled axon from the arbors all the remaining axons that expressed only CFP (**Fig. 3a–d**).

At the site of nerve crush, the several CFP-expressing axons and the single YFP-expressing axons lost all fluorescence, indicating that the crush had damaged all the axons in the bundle (**Fig. 3e**). Several days later, after all the axons regenerated across the crush site, the distribution of CFP-labeled axons showed that the whole branching pattern of the nerve was reconstituted (**Fig. 3f** and **g**). Because all

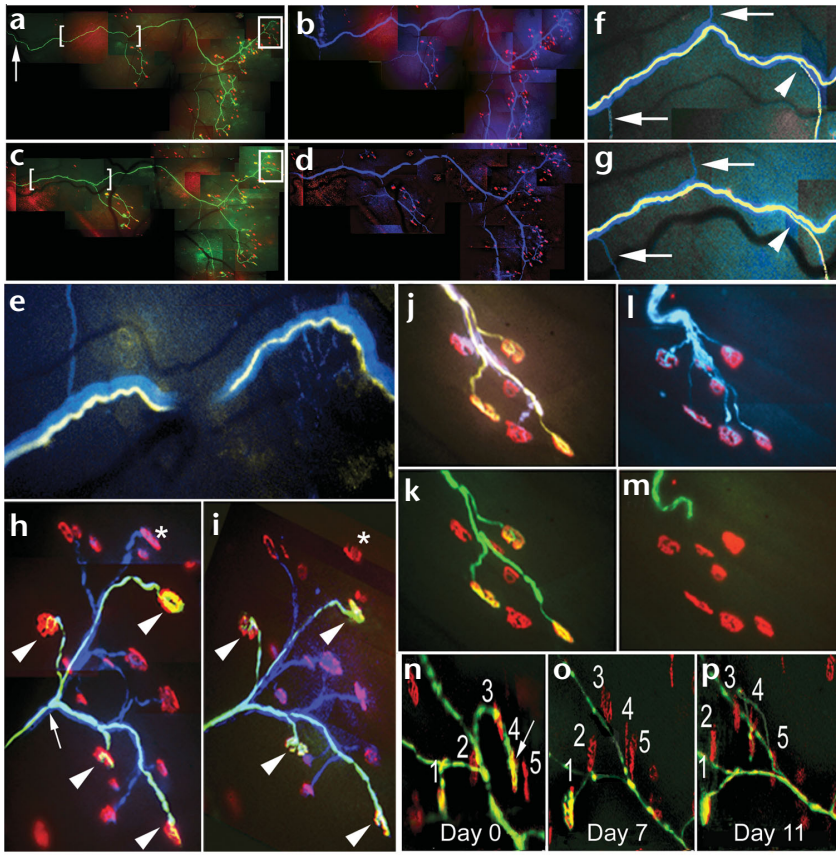

Fig. 3. Axonal regeneration assessed in double-transgenic mice, in which all axons are labeled with CFP (blue) and a single axon is also labeled with YFP (green when shown alone in **a**, **c**, **k**, **m**–**p** and yellow when superimposed on the CFP image in **e**–**j**). (**b**, **d**, **l**) Images of CFP alone. Synaptic sites labeled with rBTX are red. (**a**–**d**) Field imaged before (**a**, **b**) and 25 days after nerve crush (**c**, **d**). (**e**–**i**) High-magnification views of areas (in **a** and **b**) indicated by arrow (**e**), brackets (**f**, **g**) and boxes (**h**, **i**). Crush site, to show that all axons were transected. (**f**, **g**) Nerve trunk, to show that the YFP-labeled axon bypassed two potential branching points (arrows) before bifurcating at the site where it had branched initially (arrowhead). (**h**, **i**) Cluster of synapses showing that the YFP-labeled axon reinnervated all four of its original targets (arrowheads), but that one of the sites originally occupied by a blue axon (asterisk) remained denervated. Arrows (in **h**) show a YFP-labeled axon bifurcating at two successive nodes of Ranvier, identified by marked reduction of axonal caliber. (**j**–**m**) From another animal, before (**j**, **k**) and 25 days after (**l**, **m**) nerve crush. In this case, the YFP-labeled axon approached the muscle fibers but did not reinnervate three original targets, all of which were reoccupied by blue axons. (**n**–**p**) Multiple views of a single YFP-labeled axon. This axon originally innervated site 4 (arrow in **n**). After axotomy, it initially grew past the branch point leading to this site (**o**), but subsequently extended into this branch and reinnervated site 4 (**p**).

branches in the nerve were seen by CFP axon labeling, it became clear that each regenerating YFP-labeled axon not only projected into the branches that it had occupied formerly, but also bypassed the numerous branches that it had not previously innervated (**Fig. 3f** and **g**; **Table 2**, axons 10–12). In most cases, only a single axon was present in each endoneurial tube (assessed by light microscopy). In addition, only one axon occupied each neuromuscular junction, indicating that the YFP axon not only found most of its original targets but was also the only axon to successfully reinnervate them (**Fig. 3h** and **i**).

Of the small proportion of synaptic targets that were not reinnervated by the original YFP-labeled axons (**Table 1**, lines 10–12), approximately half of these muscle fibers (54%) were now innervated by a different axon (CFP-labeled rather than YFP-labeled; **Fig. 3h**–**m**), and 26% were not reinnervated by any axon as evidenced by a vacant AChR site (asterisk, **Fig. 3h** and **i**). In one of these latter cases, a vacant site was eventually innervated by the original axon (**Fig. 3n**–**p**), suggesting that some of these errors might have disappeared had we monitored all the muscles at later time points. The remaining 20% of missing branches were explained by the degeneration of some target muscle fibers (the AChR site was no longer present). It is likely that these missing fibers were damaged during the first time point axon mapping and nerve crush. In thicker muscles, fibers remain intact and can be re-identified throughout the life of the animal, suggesting that muscle fiber turnover is not common[31].

The YFP/CFP mice provided two additional insights into the mechanisms by which the axons reach their targets. First, when an 'incorrect' axon occupied a synaptic site, its entry into that site was invariably from the same direction as the original axon (**Fig. 4**). This suggests that pre-formed pathways guide axons all the way to synaptic sites and that these paths may be used by any axon that happens upon the path. Second, although branching could occur by bifurcation of growth cones, some branches were apparently initiated as collaterals from axon shafts (**Fig. 3n**–**p**). This behavior resembles the 'collateral sprouting' thought to underlie many instances of axonal branching in the central nervous system[32,33].

Association of axonal branches with nodal sites

Axonal branch points in normal nerves occur at nodes of Ranvier[23]. We identified nodes in live mice by a marked reduction in axon caliber (arrow in **Fig. 3h**) and, as expected, branch points occurred at nodes. We also found that new branches in regenerating axons occurred at sites where nodes had previously been. In the rare instances when a regenerating axon left its original pathway, the novel sprout also originated from the site of a previous node (**Fig. 4**). Likewise, entry into novel pathways occurred at previous nodes (blue arrowhead in **Fig. 4a** and **b**). Together, these results show that nodes of Ranvier have unexpected specialized properties. Nodal sprouting of intact, adult axons also occurs at nodes of Ranvier[33], but this has been interpreted to mean that axonal growth is constrained by the myelin that covers internodes. The fact that regenerating axons sprout at former nodal sites after myelin and axons have degenerated, however, suggests that at least some specializations are associated with the Schwann cell basal lamina. In this respect, nodal basal lamina might resemble the basal lamina of the synaptic cleft, which contains localized cues that are recognized by regenerating axons[27].

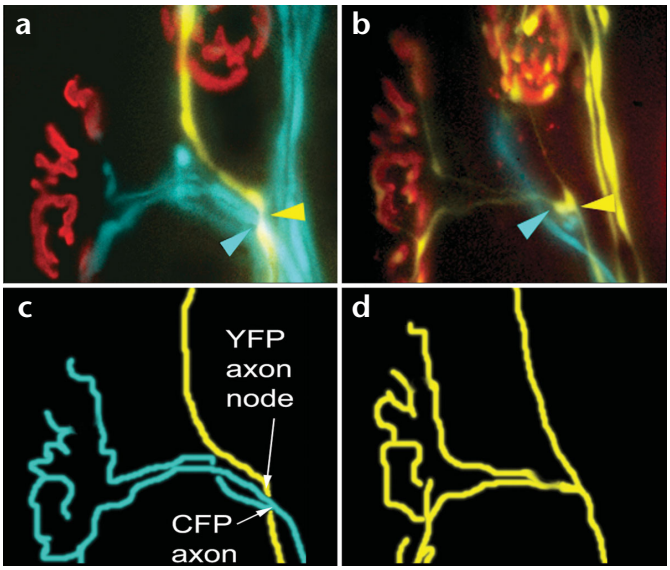

Fig. 4. Regenerating axons exit and enter Schwann cell tubes at former nodal sites. Before injury (**a**, sketched in **c**), a YFP-labeled axon innervated a neuromuscular junction above this panel. Seven days after axotomy (**b**, sketched in **d**), this axon had regenerated, bifurcated, and left its own tube at the site of a previous node of Ranvier (yellow arrowhead). The escaped branch used another nodal site (blue arrowhead) to enter a nearby tube originally occupied by a CFP-labeled axon. Once the yellow axon was in the CFP axon's tube, it reinnervated the synaptic site formerly occupied by the CFP axon.

Stereotropic guidance by endoneurial sheaths

Growth through endoneurial sheaths presumably involves molecular interactions between growth cones and guidance molecules, but the sheaths may also act as patent (unobstructed) tubes to mechanically confine axons. To investigate this, we took advantage of the fact that a transient stage in the degeneration of axons is the formation of fragments (20–100 μm in length) contained within the endoneurial tubes[30]. We first visualized YFP-labeled axons at bifurcation points and then reimaged these sites after axotomy when fluorescent axon fragments were numerous. By trapping this region of the muscle between a cover slip and supporting plate, we exerted direct pressure on the endoneurial tubes, causing the YFP-labeled fragments to move in an anterograde or retrograde direction depending on the direction of the pressure (**Fig. 5**). The fragments always remained in the tubes, and could be pushed back and forth many times. Furthermore, the fragments could be pushed into either branch of a previous bifurcation point. This finding suggests that endoneurial tubes provide mechanical constraints, and that guidance through them does not depend entirely on growth cone navigation.

Nonselective reinnervation by cut nerves

Finally, we considered a possible discrepancy between our results and previous reports of regenerating motor axons that show limited (if any) synaptic specificity for individual fibers within a muscle[14,15,34,35]. One difference between the previous and present studies is that we minimized trauma and speeded reinnervation by crushing rather than cutting the nerve. We therefore monitored reinnervation after cutting the nerve and, as expected[24,25], this procedure delayed reinnervation (data not shown). Furthermore, the specificity of reinnervation was markedly degraded after nerve cut (**Fig. 6a** and **b**). First, fewer synaptic sites were reinnervated after cut compared to crush, perhaps because some axons did not regenerate, regenerated more slowly, or degenerated after axotomy. Muscle fibers, and synaptic sites on them, were markedly atrophic in such denervated areas. Second, even in areas that were mostly reinnervated, the incidence of errors was substantial: >90% of synapses made by YFP-labeled axons were on muscle fibers previously innervated by other axons (compared to <5% after nerve crush). This degradation of specificity may result from misrouting of axons at the site of trauma, and consequent entry into incorrect endoneurial tubes or even growth outside of endoneurial tubes. Even axons that retraced original pathways for long distances often made aberrant turns at distal branch points, far from the site of injury. In one case (**Fig. 6**), the labeled axon re-branched at a point ~3 mm distal to the nerve cut (pink arrows in **Fig. 6a** and **b**) to reinnervate some muscle fibers that it had originally innervated plus some fibers that it had not originally innervated (**Fig. 6c** and **d**). This result suggests that trauma at the cut site may not be the only detriment to specificity; delay *per se* may also be involved. The integrity of the endoneurial and perineurial tubes may decrease gradually after denervation, resulting in blockade of some axons and facilitated escape of others.

Discussion

We have used a new *in vivo* imaging strategy to assess, for the first time in any vertebrate, the cellular specificity of synapse forma-

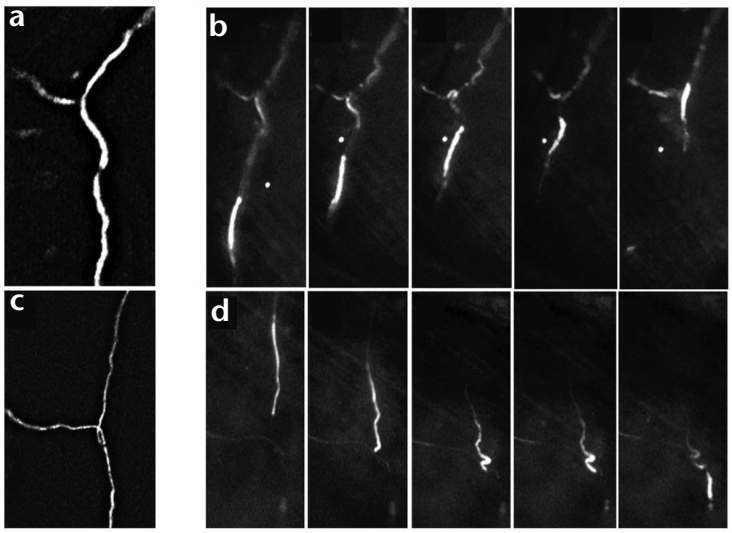

Fig. 5. Fragments of degenerating axoplasm reveal the patency of endoneurial tubes. (**a**, **c**) Two axonal segments fluorescently labeled with YFP at bifurcation points before axotomy. (**b**, **d**) Series of micrographs taken at ~5-s intervals (left to right), several hours after axotomy. YFP-labeled fragments were pushed anterogradely (**b**) and retrogradely (**d**) through the endoneurial tubes as pressure was gradually applied to the muscle.

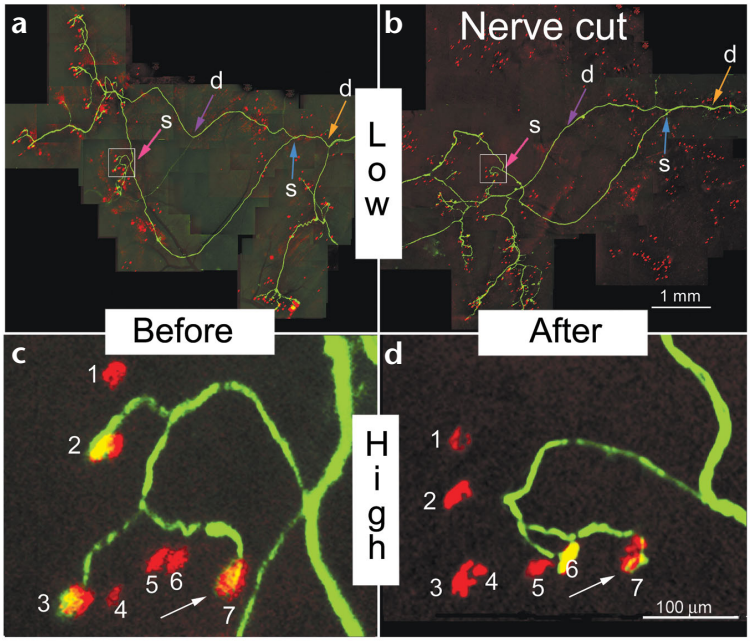

Fig. 6. Poorly selective reinnervation of original targets after nerve cut. The field was imaged before (**a**, **c**) and 25 days after (**b**, **d**) nerve cut. (**a**, **b**) Colored arrows show corresponding branching points that are the same (s) and different (d). (**c**, **d**) Boxed regions are shown at higher magnification. In this region, the YFP-labeled axon reinnervated only one of its three original targets (endplate 7, arrow), and also innervated one novel target (endplate 6).

tion during recovery from nerve injury. When trauma to the nerve was minimized, individual axons made most of their new synapses on exactly the same cells they had originally innervated. Thus, the motor unit—the axon and the cohort of muscle fibers it innervates—was substantially reconstituted. When greater trauma accompanied axotomy, specificity was dramatically degraded. The differing results from nerve crush and cut correspond to the distinct clinical outcomes seen after compression versus transection of peripheral nerves in humans[36].

Neural specificity arises from the guidance of axons to their targets and the recognition of those targets by the axons[37,38]. In principle, the reconstitution of motor units might involve either or both of these processes. For example, molecules exist that bias neuromuscular synapse formation in favor of appropriate partners[39], and these could convert a specific subset of nerve–muscle contacts into functional connections. We found, however, that regenerating axons retraced former pathways and branched at original branch points even when they were thousands of microns from their targets, suggesting that the specificity seen here results from pathway guidance rather than target recognition. This guidance, in turn, could reflect either specific chemical matching of particular axons to particular endoneurial tubes, or more generic cues, that guide any axon in the vicinity. We cannot fully distinguish between these alternatives, but several observations, including the accurate retracing of one axon's pathway by another axon (**Figs. 3** and **4**), support the latter explanation.

What, then, are the generic cues that direct extension and branching? Early studies of axonal guidance[23,40] emphasized roles of mechanical discontinuities, whereas more recent work focuses on identification of molecular cues along the pathway that growth cones recognize[37]. We suggest that both mechanical and molecular factors are involved. For example, there are numerous surface-associated, growth-promoting proteins in peripheral nerves, such as laminins in the basal lamina and L1/NgCAM in Schwann cells[41,42]. Here we have shown that endoneurial tubes can also provide strictly mechanical guidance (**Fig. 5**). This passive component may contribute, along with molecular growth-promoting factors, to axonal guidance during peripheral regeneration. Likewise, axons might sometimes branch passively when the opportunity arises, but might also encounter localized branch-inducing cues. In fact, proteins such as Slit and the neurotrophic factor GDNF are known to act on motor axons and can promote branching[43,44]. It will be interesting to determine whether such factors are concentrated at the nodal basal lamina, in light of the current findings which suggest specialized properties of these cells.

When nerves were cut rather than crushed, specificity was dramatically degraded both at branching points and at specific synaptic connection points. This difference has been noted previously, both in animals and in humans[23–26,35,36,45], but has generally been attributed to barriers or misrouting at the site of injury. Our observation that axons sometimes crossed the cut readily, only to commit errors far distally, suggests that the delay also undermines specificity. In fact, the cellular and molecular environment in and around the nerve stump changes over a prolonged time course after axotomy. For example, macrophages that invade to remove axonal debris also release neuroactive cytokines and disrupt the basal lamina[46]. Likewise, Schwann cells and fibroblasts proliferate[47] and begin to synthesize new trophic factors, adhesion molecules and proteases[41,42]. Thus, tubes may become more porous after injury, and axons may encounter attractants and repellents that tempt them to grow in new directions. Such changes are generally evaluated in terms of their positive or negative effects on the number of regenerating axons[36], but our results indicate that they may also have biologically and clinically important effects on the accuracy with which axons reconstitute their original innervation patterns after injury.

Methods

Animals. Generation and characterization of thy1-YFP-H, thy1-YFP-F, and thy1-CFP-D mice are as previously reported[18,20] (strain YFP-H was from Jackson Laboratories, Bar Harbor, Maine, as are strains YFP-16 and CFP-23, whose properties are similar to those of YFP-F and CFP-D, respectively.) To construct the transgene, 'humanized' CFP or YFP cDNAs were linked to a fragment of the mouse *thy1* gene that promotes neuron-specific expression. In most lines, including YFP-F and CFP-D, all motor axons were intensely labeled in postnatal animals. In a few lines, including YFP-H, only a subset of motor axons was labeled. The precise number of labeled axons per muscle varied from animal to animal, but was 0–2 in the regions of the platysma (ventromedial) or sternomastoid (superficial) that we used. All lines were maintained on a non-Swiss albino background, chosen for its heartiness. Double-transgenic mice were produced by mating the lines, and genotypes were assessed either optically or by PCR.

Imaging. Experiments were done on the sternomastoid or platysma muscles of the neck. Protocols for use of living mice were approved by the Animal Studies Committee at Washington University School of Medicine. Mice were anesthetized and mechanically ventilated. Muscles were exposed by a ventral midline incision, which was superficial for the platysma, but subcutaneous fat and salivary glands were retracted to expose the sternomastoid.

Muscles were bathed for 1 min in rhodamine-α-bungarotoxin (rBTX, 5 μm), and images were taken under low-light fluorescence using a silicon-intensified (SIT) camera. Muscles were then denervated by crushing the nerve for 5–20 s with #5 forceps, or by cutting the nerve with iridectomy scissors. After imaging, the skin incision was closed, and the animal was returned to its cage. One to 25 days after axotomy, muscles were re-exposed and re-imaged. The dose of rBTX we used did not lead to paralysis during reinnervation. At least 70% of AChRs must be blocked to block muscle activity, and our procedure blocked <60% initially and then was reduced to <30% by the time the first axons returned[48,49]. Montages were constructed from separate images using Adobe Photoshop. Surgical methods and imaging protocols were done as previously described[21,22].

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Competing interests statement

The authors declare that they have no competing financial interests.

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