
Review

False Resurrections: Distinguishing Regenerated from Spared Axons in the Injured Central Nervous System

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ABSTRACT

Several recent studies report that axon regeneration can be induced in the mature mammalian nervous system by novel treatments or genetic manipulations. In assessing these reports, it is important to be mindful of the history of regeneration research, which is littered with the corpses of studies that reported regeneration that later proved incorrect. One important reason is the “spared axon conundrum,” in which axons that survive a lesion are mistakenly identified as having regenerated. Here, we illustrate the problem and propose criteria that may be used to identify regenerated vs. spared axons, focusing on the injured spinal cord. *J. Comp. Neurol.* 459:1–8, 2003. © 2003 Wiley-Liss, Inc.

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On the face of it, a study of axon regeneration in the central nervous system would seem simple to perform. One simply cuts or otherwise damages a population of axons, and then evaluates whether those axons re-grow. In a typical experiment involving spinal cord injury, for example, one would produce a lesion in the spinal cord, wait for some period of time to allow for possible axon regeneration, and then trace particular spinal tracts by using tract-tracing techniques. Numerous studies indicate that there is minimal axon regeneration in normal animals (Schwab and Bartholdi, 1996). The axons that had been cut retract for some distance from the injury and persist as retraction balls, perhaps exhibiting regenerative sprouting into nearby territory. In contrast, several recent studies report that, in animals that receive some treatment or that carry a mutation in a gene that presumably encodes an inhibitor of axon growth, the axons that had been cut regenerate around, beyond, or sometimes even through the lesion site.

What could possibly go wrong in such a simple experiment? The answer is that axons are remarkably resilient and can survive displacement and stretch. Because of this resiliency, axons that are revealed by tract tracing at some time point after a lesion may not have been cut in the first place, and treatments or genetic manipulations may re-

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sult in an increased number of spared axons in the experimental group. This potential problem is exacerbated by the fact that many recent studies have adopted surgical approaches that are designed to minimize physical damage, to lower the bar for successful axon regeneration. In what follows, we consider the experimental paradigms and their attending risk for false-positive results.

MAXIMIZING THE CERTAINTY THAT AXONS ARE CUT

If there is an issue of possible axon-sparing, why not just do the lesion in a sufficiently aggressive way to make absolutely sure that the target axon population is completely cut? For example, for a study of axon regeneration in the spinal cord, one approach is to completely transect the cord, lifting up the cut ends to be sure that the transection is complete. The problem is that after complete surgical transection of the spinal cord, the two ends retract apart, leaving a fluid-filled space of varying length. Secondary degeneration of tissue surrounding the injury site then ensues, leading to a further separation of the cut ends. Over time, a glial/connective tissue scar forms around the cut ends, and in most animals (except mice), a loose network of scar tissue develops within the lesion site leaving the two ends of the cord separated by many millimeters. Mice exhibit a somewhat different postinjury response in which the lesion site in the spinal cord is filled-in by a connective tissue matrix (Zhang et al., 1996). This matrix then contracts over time, drawing the two ends of the spinal cord closer together in a way that resembles the wound healing process seen in skin and other tissues.

Obviously, complete transections create a tissue environment that is inimical to axon regeneration. In species other than mice, the physical distance between the cut ends alone is daunting, to say nothing of the fact that the lesion site contains little if any structural matrix that could support a growing axon. Even in mice, where the two ends of the cord are drawn closer together, the lesion site is filled in with a dense connective tissue scar, which is presumably a hostile environment for central nervous system (CNS) axons. Accordingly, studies that document successful axon regeneration after complete transections are especially noteworthy (Ramon-Cueto et al., 1998). Seeing even a few labeled axons extending through the non-CNS tissue environment of the lesion site created by a complete transection is evidence that these axons in fact regenerated and were not spared. Hence, we come to the first criterion that supports the interpretation that a given axon has regenerated: *(I) that the axon extends from the CNS into a non-CNS environment, specifically, the tissue environment of the scar that develops at the injury site.*

Of course, the above criterion is useful in any spinal cord injury experiment, including those involving partial injuries, and there are recent studies that report this type of regeneration after partial transection injuries (Neumann et al., 2002; Qiu et al., 2002). In studies involving partial injuries, however, it is important to consider the possibility of spared axons (see below). The key here is demonstrating that the putative regenerating axons actually grow into a non-CNS tissue environment. Also, with partial injuries, there is a possibility that axons are pushed from their usual position so as to become embedded in areas in which scar tissue later develops. This

mechanism may be especially problematic in mice, where a dense connective tissue matrix forms at the injury site and also in areas that contain dense degeneration debris (for example the dorsal columns; more on this below).

Complete transections are used most commonly by investigators who are interested in studying the possible regeneration-promoting effects of various grafts and transplants (see for example (Ramon-Cueto et al., 1998; Coumans et al., 2001). The reason is twofold: (1) A complete transection provides the greatest certainty that the target axon populations are actually interrupted; and (2) the complete transection creates a physical space into which a graft or transplant can be placed. Because it is the graft or transplant itself that is the potential enhancer of host axon regeneration, successful regeneration is reflected by growth of host axons into the graft or transplant. This growth represents the second definitive criterion that can be used to identify a regenerated axon: *(II) that the axon extends from the host CNS into a nonhost graft or transplant.* To definitively establish that regeneration occurred in this setting, it is critical to definitively document the boundaries between the host CNS and non-host graft or transplant, to establish that the CNS axon actually extended beyond the boundary into the nonhost tissue environment.

LOWERING THE BARRIER FOR REGENERATION BY MINIMIZING TISSUE DAMAGE

Because the lesion that results from a complete transection creates such a hostile tissue environment, lesion paradigms have been developed that minimize the physical damage to the cord and the resulting cavitation and physical separation. The current “industry standard” involves partial transections of one sort or another, in which the lesion is designed to selectively interrupt certain pathways but where a tissue bridge holds the proximal and distal ends of the cord together, maintaining tissue continuity. Examples of such partial transactions include dorsal hemisections, used extensively in recent studies of corticospinal tract regeneration, dorsolateral quadrant lesions that interrupt the rubrospinal tract, and lateral hemisections (or over-hemisections), that interrupt all tracts on one side but spare some or all tracts on the opposite side. It is in these partial transection paradigms that the possibility of axon-sparing arises, with the perpetual question being whether a given axon was actually cut in the first place.

EXAMPLES OF AXON SPARING IN PARADIGMS DESIGNED TO INTERRUPT THE CORTICOSPINAL TRACT

The problem of axon-sparing can be well illustrated by examples involving the corticospinal tract (CST). The main component of the CST of rodents is located in the ventral part of the dorsal column (Fig. 1). A few CST fibers also descend in the lateral column, especially in cervical regions, and in the ventral column (the ventral CST). A popular surgical paradigm for studying factors that may induce regeneration of the CST is the “dorsal hemisection,” which is designed to cut the main CST bilaterally while producing minimal overall tissue damage. Transec-

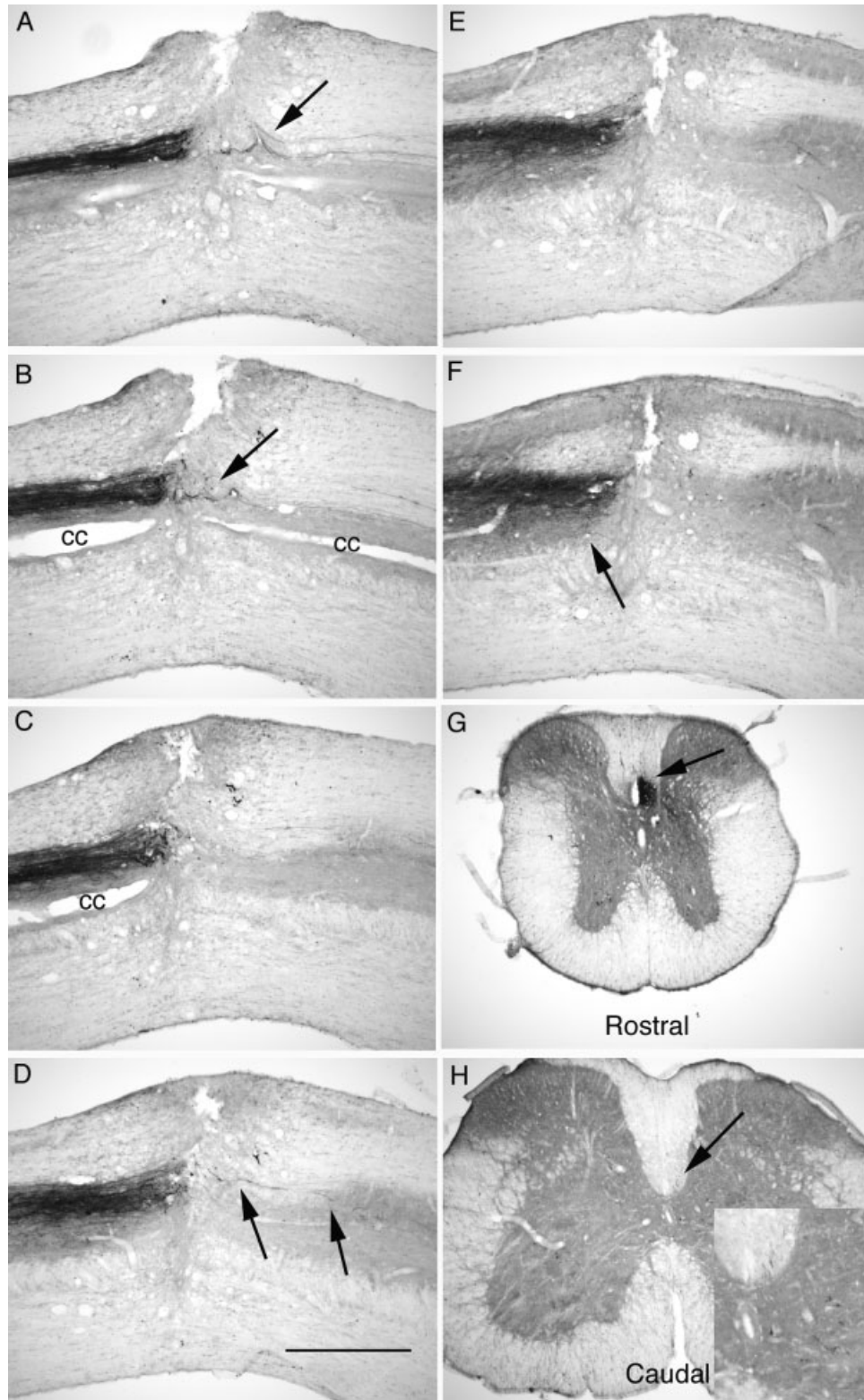


Fig. 1. Stretched but still patent axons after corticospinal tract (CST) transections produced in such a way as to minimize tissue damage. **A-F**: Serial sagittal sections from a control mouse prepared as part of a study of the effects of mutations of *Nogo* on the regenerative potential of CST axons (Zheng et al., manuscript submitted for publication). A dorsal hemisection was made at T8 by penetrating the dura mater with a 30-gauge needle and then inserting a micro-knife that was moved back and forth to transect the dorsal columns bilaterally. Fluoro-Ruby-conjugated BDA was injected into the right sensorimotor cortex during the same operative procedure (two injections of 10% Fluoro-Ruby BDA placed at 1.0 mm lateral, 0.5 mm deep to the cortical surface, and 0.5 mm anterior and posterior to bregma, respectively). The mouse was allowed to survive for 17 days and was then perfused with 4% paraformaldehyde (see text for additional methodological details). Arrows indicate extremely distorted (stretched) axons

at the injury site and labeled axons in the dorsal column caudal to the injury site. **G,H**: Transverse sections approximately 0.5 mm rostral and 0.5 mm caudal to the injury site, respectively. The inset in H illustrates a few surviving labeled axons in the CST and collaterals in the gray matter. Methods: The spinal cord was removed, and a 1-cm block was taken that extended 5 mm rostral and 5 mm caudal to the center of the injury. This block was sectioned in the sagittal plane, collecting every section. The rostral and caudal segments were embedded and frozen separately and were sectioned in the transverse plane. Floating sections were washed three times in 1× PBS and 0.1% Triton X-100, incubated with ABC reagent (Vector Laboratories) overnight, washed again three times in 1× PBS, and then reacted with 3,3'-diaminobenzidine (Sigma) in 50 mM Tris buffer pH 7.6, 0.024% hydrogen peroxide, and 0.5% nickel chloride. Scale bar = 500 μ m.

tions are often made by using iridectomy scissors or a micro-knife, with the goal being to create a lesion that transects the cord down to the level of the central canal. Thus, a successful dorsal hemisection would cut all CST fibers except those in the ventral CST.

When a dorsal hemisection is done without any “special” surgical techniques (see below), the tissue surrounding the transection often degenerates and/or pulls apart, leaving a V-shaped lesion site in the dorsal part of the spinal cord. In rats, this V-shaped lesion is usually a cavity that may contain loose scar tissue. The scar tissue in the lesion site may be lost during dissection or tissue processing, especially when floating sections are processed, so that the lesion appears as a V-shaped hole. When CST axons are then traced by using tract-tracing techniques, labeled axons extend to within a few hundreds of micrometers of the lesion margin, ending in the highly characteristic retraction balls that were beautifully described by Ramon y Cajal in studies carried out almost a century ago (Ramon y Cajal, 1959). In the absence of some transplant or tissue bridge to fill the lesion cavity, cut CST axons could regenerate by (1) growing through the lesion site, (2) growing along the margin of the lesion, (3) growing into and along the gray matter ventral to the lesion margin, (4) growing laterally and then along the surviving white matter in the surviving ventral part of the lateral column, (5) diving ventrally (through the gray matter) and growing in the ventral column.

In mice, it is possible to produce dorsal column lesions that do not lead to the development of a V-shaped lesion by producing lesions in a way that causes minimal damage to the dura mater. For example, Figure 1 illustrates a case in which a dorsal column lesion was created by using a 30-gauge syringe needle to penetrate the dura mater and then carefully inserting a thin micro-knife, which was then moved back and forth in an arc. In Figure 1, which illustrates the appearance of the lesion site 17 days after lesion, the two ends of the cord are separated by a very thin region of scar tissue. The presence of a tissue bridge at the transection site produces another possible route for regenerating fibers to take—directly through the scar tissue.

The important point is that, although the surgical approach described above is designed to transect all CST axons, some axons may be stretched and displaced but remain intact. Such axon-sparing may only become apparent when a thorough serial section reconstruction is carried out. For example, in the case illustrated in Figure 1, the lesion appears complete in most sections, but serial sections reveal that a few axons continue past the lesion site. The image of the labeled axons as they pass through the lesion site suggests that the axons were severely stretched and/or displaced by the micro-knife during lesion production (Fig. 1A,B,D). In the caudal segment, the same axons continue in their normal location in the CST in a straight and unbranched trajectory (Fig. 1D, arrows). That these axons are straight and unbranched in the caudal segment distal to the injury strongly suggests that the axons are spared rather than regenerated.

The surgical paradigm used in the experiment of Figure 1 was designed to produce minimal tissue damage so as to create an environment that would be less inimical to regeneration. It is likely that the probability of axon-sparing increases in direct proportion to the extent of tissue-sparing as a result of the use of special lesion techniques.

AXON STRETCHING, FUNCTIONAL COMPROMISE, AND RAPID RECOVERY OF FUNCTION

It would certainly not be surprising if action potential transmission was transiently disrupted in axons that were stretched in the manner suggested in Figure 1. Axonal transport mechanisms might also be transiently disrupted, although the transport of biotinylated dextran amine (BDA) into distal segments indicates that transport is restored by the time the BDA reaches the affected segments. Importantly, disruption of action potential transmission in stretched axons could account for transient loss of function at early postinjury intervals. Similarly, recovery of the ability to transmit action potentials past the site of stretch could account for the rapid recovery of function that is often seen within the first few days after a spinal cord injury. It must be kept in mind that any enhancement of early recovery of function as a result of some treatment could be a result of restoring transmission along these stretched but physically intact axons.

IMAGES SUGGESTIVE OF REGENERATION

Claims for CST regeneration in recent studies that have used partial lesion paradigms have been based on several types of evidence. One is the extension of axons from the collection of retraction balls ventrally and caudally through the gray matter (mechanism 3 above). Of course, axons normally branch off from the CST all along its course to dive into the underlying gray matter; therefore, it is important to distinguish the normal collaterals from the putative regenerating axons. In this regard, criteria that support the identification of particular axons as regenerated are (A) That the putative regenerated axons arise from near the site of amputation (i.e., in or near the collection of retraction balls in the dorsal column). It would be very unlikely to label selectively, by chance, exactly the population of axons that diverged from the CST at the level of the injury. Seeing a large number of axons extending ventrally and caudally near the point of amputation is suggestive of regenerative growth or at least extensive collateral sprouting. (B) That the putative regenerated axons extend caudally through gray matter rather than through white matter. Most of the gray matter collaterals of CST axons extend in the transverse plane and do not extend for long distances longitudinally through the gray matter. For example, after large tracer injections into the motor cortex, collaterals of the CST can be seen in the gray matter throughout the rostrocaudal extent of the spinal cord. Most of these fibers take a more or less perpendicular course as they leave the main tract, and ramify within the segment. Thus, when a lesion transects the dorsal column and the descending CST axons, the labeling of gray matter collaterals stops abruptly within a few hundreds of micrometers from the collection of labeled retraction balls (see Fig. 1D–F).

One recent study that reports regeneration makes this claim on the basis of the existence of labeled CST axons that fan out from the point of transection, and then extend longitudinally through the gray matter at or below the level of the lesion (Bradbury et al., 2002). Images of this sort are especially suggestive of regeneration because the putative regenerated axons fan out in an unusual trajec-

tory from their point of origin near the retraction balls in the main CST in the dorsal column. Thus, this report illustrates two additional criteria that can be used to identify a regenerated axon: (III) *that the putative regenerated axon originates at or near a site of amputation; and* (IV) *that the putative regenerated axon takes an unusual course through the tissue environment of the CNS.*

One can be fooled, however, if one restricts one's examination to single sections. For example, if longitudinal sections are not perfectly oriented, the labeled CST may appear to end a segment or two rostral to the labeled collaterals in the gray matter (depending on how far off the plane of section actually is). Such an image may be interpreted as indicating extensive sprouting. This can be determined easily by examining serial sections.

Other recent studies provide a different type evidence to support an interpretation for regeneration—the presence of labeled axons in the dorsal column below the level of the injury. The key here is a comparison between control animals and experimental animals that have received some treatment. In one study, animals were treated with enzymes that degrade chondroitin sulfate proteoglycan (Bradbury et al., 2002). In another study, the treatment was a combination of olfactory ensheathing cells and methylprednisolone (Nash et al., 2002). In a third, the treatment involved delivery of a peptide that is an antagonist to the Nogo-66 receptor (GrandPre et al., 2002). In each study, experimental animals had more labeled CST axons caudal to the injury than control animals with identical injuries. Data of this sort, taken by themselves, are especially vulnerable to the “spared axon” problem. Figure 1 illustrates an example of what can happen. Here, the lesion extends through the entire dorsal column and, in some sections, appears complete. Careful reconstruction of the lesion site and the labeled CST through serial sections reveals axons that extend past the lesion and continue in the caudal dorsal column. These axons can be seen in the transverse section caudal to the lesion in the ventral-most portion of the dorsal column (Fig. 1H). If one did not carefully reconstruct the lesion site in serial sections, spared axons might be interpreted as having regenerated. Stated another way, one cannot be sure that any CST axons seen below the level of the injury were actually cut in the first place.

One could argue that this possibility is made less likely by the statistical comparison between control and treated groups that reveal differences in the numbers of labeled axons in animals with similar injuries. The problem, however, is that the treatment or genetic manipulation may result in a greater degree of axon-sparing, or an enhanced survival of axons that are stretched and displaced. Such a phenomenon could also lead to enhanced recovery of function (see below). The key point is that a claim that labeled axons in *their normal location* in the CST are regenerated must be considered provisional, unless the possibility of axon-sparing is eliminated. At a minimum, it would seem prudent to carefully document the course that axons take past the lesion site to provide some other evidence that they are regenerated and not spared (for example, that the axons grow through the lesion site or around it taking an abnormal route through the tissue environment). This approach in fact was done in the experiments involving injections of chondroitinase (Bradbury et al., 2002), in which labeled axons were seen along the margin of the lesion (mechanism 2 above). Unfortunately, however, the

complete trajectory of the labeled axons was not assessed in serial sections; therefore, their actual origin and course could not be determined. In particular, it was not established whether the axons that were seen in the dorsal column in caudal segments were the continuations of axons that extended along the lesion margin or instead were axons that might have been spared.

An important point is that, if axons that extend into caudal segments are spared, they should be present in their normal locations (in the ventral part of the dorsal column in the case of CST axons, see Fig. 1H). In this regard, it is noteworthy that in the study of the effects of peptide antagonists to Nogo-66, the evidence was somewhat different, in that labeled axons were found in unusual locations in the white matter (Bradbury et al., 2002). This finding makes it unlikely that the labeled axons were spared fibers that survived the lesion.

It might be argued that the criterion requiring an abnormal course is unreasonably stringent. Is it possible, for example, that some treatment or genetic manipulation will make it possible for axons to grow through or around a lesion site and then re-enter their normal tract caudal to the injury? Of course regeneration researchers hope for this result. Nevertheless, the possibility of axon regeneration after spinal cord injury is too important to fail to consider alternatives, such as the axon-sparing illustrated in Figure 1. One can even imagine a situation in which axons are displaced into abnormal territory, so that it would appear that they had grown there during regeneration. Thus, even when axons appear to have an abnormal trajectory through the lesion site, this finding is not definitive evidence that they are regenerated.

TIME AND DISTANCE: WHAT IS REASONABLE?

Under conditions that are optimal for axon growth (for example, during normal development), axons grow at a rate of approximately 1 mm per day. This is likely to be the maximal rate of axon regeneration that could be obtained under the best of circumstances after axon amputation in adult animals. It also seems reasonable to assume that it would take time, under the best of circumstances, for an amputated axon to recover from injury and begin to extend. Putting a number to this requires speculation, but a few days seems to be a reasonable assumption for the minimum time required to convert to a growth mode, especially if it is necessary to up-regulate the expression of growth-associated genes. It also seems reasonable to expect that a growing axon would be delayed somewhat as it circumnavigates the lesion site or grows through the relatively nonpermissive environment at and around the injury site.

Obviously, the best solution to this problem would be to develop techniques to visualize the response of living axons after injury in vivo. This has been accomplished for axons of the peripheral nervous system (Nguyen et al., 2002) but, to date, has not been achieved for spinal cord axons. Until it is possible to directly visualize amputated and potentially regenerating spinal cord axons in vivo, we must rely on the standard anatomic approach of inferring what the rate of growth must be by comparing axon distribution in different animals that have been killed at different postinjury intervals. So far, this has not been done in any of the recent experiments that describe treat-

ments or genetic manipulations to enhance axon regeneration in the spinal cord. Absent definitive data, it seems reasonable to expect that it would require many days for an axon to regenerate through or past a lesion site and extend for any distance through parenchyma on the other side. Accordingly, if one produced a lesion of the CST and then assessed CST axon distribution during the first few days after the injury, there should be *no* labeled CST axons extending beyond the level of the injury. If regeneration did occur, there should be a gradual advance of the labeled axons over time. Thus, another criterion for identifying regenerated axons is *(V) regenerated axons should extend no further than could be accounted for by plausible regeneration rates*. If labeled axons are seen extending for long distances distal to an injury at early postlesion intervals, this finding would suggest that these fibers were spared rather than regenerated.

What then might be considered a reasonable expectation regarding the best possible rate of regeneration? Based on the fact that cut axons may retract for a millimeter or so, a rough estimate might be as follows:

5–7 days after injury: Recovery from injury and initiation of a growth response.

7–10 days: Regenerating axons approach the injury site.

10–14 days: Regenerating axons grow through or around the injury site.

14–21 days: Regenerating axons enter the intact tissue environment of the distal segment.

21 days and on: Regenerating axons continue to grow through the distal segment, where the maximal expected rate of growth would be 1 mm per day.

These estimates assume that there is any regeneration at all, and of course faster rates may be possible if a truly effective means is found to stimulate axon regeneration in the vertebrate CNS.

If a claim is made for long-distance axon regeneration at early postlesion intervals after some treatment, then the regeneration must be rapid and coordinated. For example, in the experiment involving treatment with a Nogo-66 antagonist peptide, putative regenerated axons were seen at a distance of 14mm distal to the lesion (GrandPre et al., 2002). This would certainly be a remarkable regenerative response. If such regeneration occurred, it should be possible to detect large numbers of regenerating axons at intermediate locations at early postlesion intervals. Indeed, regardless of the rate of growth, it should be possible to find the proper postinjury interval to catch regenerating axons as they are extending, for example, when axons have grown just past the injury site but no further. At this time, one would expect regenerating axons to have a definitive tip with some sort of growth cone-like specialization. Thus, a criterion for claiming that particular labeled profiles are in the process of regenerating is *(VI) that the axons have a morphology consistent with growth in progress*.

Claims for long-distance regeneration at any particular postinjury time point should perhaps be considered provisional in the absence of strong supporting data documenting the presence of growing axons at intermediate locations at earlier time points. This is especially true of any claim for extraordinarily rapid or extensive long-distance regeneration.

One other implication of the consideration of the minimum time required for long-tract regeneration is that any restoration of function that is due to long-distance regen-

eration should not appear until sufficient time has passed for axon regeneration to have occurred. Thus, any recovery that is seen during the first 7–10 days after a spinal cord injury seems unlikely to be due to long-distance regeneration and reconnection of amputated axons with targets on the opposite side of the lesion. For example, in the study involving Nogo-66 antagonist peptide, animals that were treated with the peptide exhibited improved locomotor function as measured by the BBB scale at the earliest postinjury period examined (4 hours after injury). The differences between treated and control groups were maintained throughout the postinjury testing period. It seems very unlikely that the very early improvement in locomotor function was due to regeneration or axon reconnection.

OTHER MORPHOLOGIC CRITERIA FOR REGENERATED AXONS

The axons that are present in the long tracts of the spinal cord are generally straight and unbranched until they give rise to terminal collaterals at their point of termination. Accordingly, when one traces the CST or other long tracts after spinal cord injury, tortuous or highly branched axons near the injury site are suggestive of regeneration. This is especially true when the axons first assume a tortuous course or begin to exhibit branching at a point just proximal the injury site. Thus, another criterion for a regenerated axon is *(VII) that the axon have a morphology that is unusual for axons of its type*. Nevertheless, tortuous trajectories, including hairpin turns, may also result from axon stretching (see Fig. 1). In this case, however, identification of the axon as spared is made on the basis that the axon has a straight and unbranched trajectory distal to the injury site.

Examples of axons with a morphology that are suggestive of regeneration are shown in Figure 2, which is a mouse in which there is a null mutation in the Nogo A gene (from Zheng et al., manuscript submitted for publication). As in the case illustrated in Figure 1, the dorsal hemisection here was created by penetrating the dura mater with a 30-gauge needle and then lowering a micro-knife into the tissue so as to transect the CST while producing minimal tissue damage. BDA was injected into the sensorimotor cortex during the same operative procedure, and the mouse was killed 17 days later. In this case, most of the labeled CST axons end in a cluster of retraction balls in the dorsal column. Serial section reconstruction reveals some axons, however, that take a meandering course through the lesion site, and then enter the dorsal-most portion of the gray matter distal to the lesion (upward-pointing arrows in Fig. 2E,F). Some of these branch extensively caudal to the lesion (upward-pointing arrows), and the collaterals then continue caudally through the dorsal-most gray matter.

So, are these regenerated or are they axons that survived the injury? The tortuous course taken by the axons through the lesion site, that they project caudally through the gray matter rather than the white matter of the dorsal column, and their somewhat meandering course might be taken as evidence that they had regenerated. At the same time, however, this animal was killed 17 days after injury; therefore, the extension of axons this far caudally would certainly represent rapid

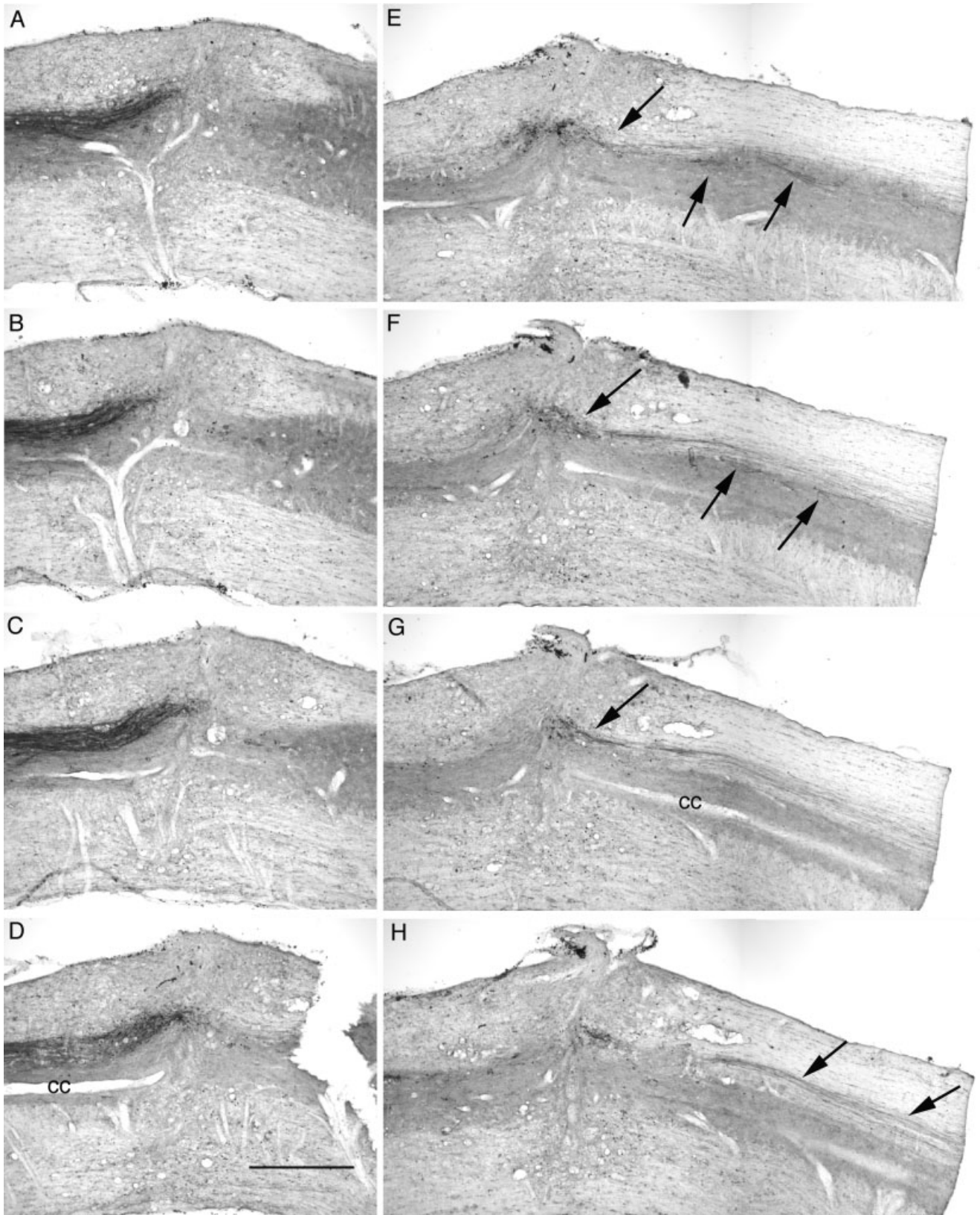


Fig. 2. Axons with unusual trajectories that are suggestive of regeneration. The panels illustrate serial sections from a *Nogo* mutant mouse prepared as part of a study of the effects of mutations in *Nogo* on the regenerative potential of corticospinal tract (CST) axons (Zheng et al., manuscript submitted for publication). The lesion and tract-tracing procedures were as described in Figure 1. Note that, in most sections, the CST appears to have been completely transected, and labeled axons end in typical retraction balls in the segment just

caudal to the transection. In some sections, however, a few labeled axons take a tortuous course through the transection site and continue in the caudal segment. Some of these dive into the underlying gray matter and continue in a somewhat meandering course, branching frequently (upward-pointing arrows in E and F). At the same time, some labeled axons can be seen in their normal location in the dorsal column, which indicate the likelihood of axon-sparing (downward-pointing arrows in panels G and H). Scale bar = 500 μ m.

long-distance regeneration. The even more critical problem with this case is that there is also evidence for spared axons. For example, in the sections illustrated in Figure 2E and F, long straight axons can be seen in the dorsal column (downward-pointing arrows). Most likely, these axons were stretched and pushed aside as the lesion was made. When there is evidence for spared axons, as in this case, other images that are suggestive of regeneration must be viewed with skepticism. The point is that one simply cannot make a definitive call based on the appearance of the axons in the absence of additional evidence. Thus, fulfilling any one or even several criteria does not necessarily establish definitively that given axonal profiles are regenerated rather than spared. Even so, some criteria are more compelling than others (see below).

NO REGENERATION WITHOUT (VISUAL) REPRESENTATION

An important implication of the above discussion is that claims for axon regeneration are difficult to substantiate without extensive documentation. It is important to trace and carefully reconstruct putative regenerated axons to obtain as many clues as possible to support the verdict that the axon is regenerated rather than spared. It is important that these supporting data be available for peer review and for consideration by potentially skeptical readers. The important practical implication is that such extensive documentation is not allowable within the space limitations of many modern high-profile journals. So, what's the answer? Should claims of spinal axon regeneration be published only in journals that permit extensive documentation? This strategy would be counterproductive given the high interest that such studies generate. One alternative might be to insist that the key supporting data be made available to reviewers and to readers. For example, such data could be made available in a Web-accessible form. For this to be useful, the data should be referenced in the text of the study and be considered as part of the review. Also, accessibility of the data should be maintained for a reasonable period to ensure reader access. Supplementary materials published online that accompany a study in a journal are well suited for this purpose as these are considered part of the paper and remain accessible indefinitely.

A SUMMARY OF THE BONA FIDES OF REGENERATED AXONS

In conclusion, we propose the following criteria for identifying regenerated axons in the injured spinal cord. These same criteria could also apply to other sites in the CNS.

(I) *The axon extends from the CNS into a non-CNS environment, specifically, the tissue environment of the scar that develops at the injury site.*

(II) *The axon extends from the host CNS into a nonhost graft or transplant.*

(III) *The axon originates at or near a site of amputation.*

(IV) *The axon takes an unusual course through the tissue environment of the CNS.*

(V) *The axon extends no further than could be accounted for by plausible regeneration rates.*

(VI) *The axon is tipped with a growth cone.*

(VII) *The axon has a morphology that is not characteristic of normal axons of its type (for example exhibiting unusual branching patterns).*

As the field moves more toward the use of minimal lesion paradigms to lower the bar for axon regeneration, it is important to increase the stringency of the evidence that we accept as indicating regeneration. Accordingly, our goal in this commentary was to set out a series of criteria that can be used to identify regenerated axons. As discussed above, some of these criteria represent definitive evidence of regeneration (I and II). The other criteria are weaker, but support the verdict that given axons are regenerated rather than spared. Obviously, the more criteria that can be met, the more secure the interpretation can be. It is our hope that setting forth these criteria will provide a useful metric for evaluating the increasing number of reports of successful regeneration after spinal cord injury.

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LITERATURE CITED

- Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB. 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416:636–640.
- Coumans JV, Lin TT-S, et al. 2001. Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. *J Neurosci* 21:9334–9344.
- GrandPre T, Li S, et al. 2002. Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* 417:547–551.
- Nash HH, Rorke RC, et al. 2002. Ensheathing cells and methylprednisolone promote axonal regeneration and functional recovery in the lesioned adult rat spinal cord. *J Neurosci* 22:7111–7120.
- Neumann S, Bradke F, et al. 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron* 34:885–893.
- Nguyen QT, Sanes JR, et al. 2002. Pre-existing pathways promote precise projection patterns. *Nat Neurosci* 5:861–867.
- Qiu J, Cai D, et al. 2002. Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron* 34:895–903.
- Ramon y Cajal S. 1959. Degeneration and regeneration of the nervous system. New York: Hafner.
- Ramon-Cueto A, Plant GW, et al. 1998. Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. *J Neurosci* 18:3803–3815.
- Schwab ME, Bartholdi D. 1996. Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev* 76:319–370.
- Zhang Z, Fujiki M, et al. 1996. Genetic influences on cellular reactions to spinal cord injury: a wound healing response present in normal mice is impaired in mice carrying a mutation that causes delayed Wallerian degeneration. *J Comp Neurol* 371:469–484.