Axonal Regeneration and Development of De Novo Axons from Distal Dendrites of Adult Feline Commissural Interneurons After a Proximal Axotomy

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Abstract

Following proximal axotomy, several types of neurons sprout de novo axons from distal dendrites. These processes may represent a means of forming new circuits following spinal cord injury. However, it is not known whether mammalian spinal interneurons, axotomized as a result of a spinal cord injury, develop de novo axons. Our goal was to determine whether spinal commissural interneurons (CINs), axotomized by 3–4-mm midsagittal transection at C3, form de novo axons from distal dendrites. All experiments were performed on adult cats. CINs in C3 were stained with extracellular injections of Neurobiotin at 4–5 weeks post injury. The somata of axotomized CINs were identified by the presence of immunoreactivity for the axonal growth-associated protein-43 (GAP-43). Nearly half of the CINs had de novo axons that emerged from distal dendrites. These axons lacked immunoreactivity for the dendritic protein, microtubule-associated protein 2a/b (MAP2a/b); some had GAP-43-immunoreactive terminals; and nearly all had morphological features typical of axons. Dendrites of other CINs did not give rise to de novo axons. These CINs did, however, each have a long axon-like process (L-ALP) that projected directly from the soma or a very proximal dendrite. L-ALPs were devoid of MAP2a/b immunoreactivity. Some of these L-ALPs projected through the lesion and formed bouton-like swellings. These results suggest that proximally axotomized spinal interneurons have the potential to form new connections via de novo axons that emerge from distal dendrites. Others may be capable of regeneration of their original axon.

Indexing terms

MAP2a/b; GAP-43; spinal cord injury

Several populations of neurons in adult and larval nervous systems have been shown to sprout de novo axons from the distal tips of dendrites following proximal axotomy. De novo axons emerging from distal dendrites were first described in axotomized anterior bulbar cells (ABCs) in the midbrain of the larval lamprey (Hall and Cohen, 1983, 1988a,b; Hall et al., 1989, 1991, 1997). Similar processes have subsequently been described in axotomized

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spinal giant interneurons of the larval lamprey (Yin et al., 1984; Mackler et al., 1986), motoneurons of cats and rats (Linda et al., 1985, 1992; Rose and Odlozinski, 1998; Rose et al., 2001; MacDermid et al., 2002, 2004; Hoang et al., 2005), retinal ganglion cells of hamsters (Cho and So, 1992), and spinal autonomic neurons of rats (Hoang et al., 2005).

Despite their unusual origin, de novo axons emerging from distal dendrites have many features that are typical of axons. They follow a tortuous path, have a uniform diameter (Hall and Cohen, 1988b; Rose and Odlozinski, 1998), and form bouton-like structures (MacDermid et al., 2004; Hoang et al., 2005). Analysis of the ultrastructure of de novo axons that emerge from the distal dendrites of lamprey ABCs shows that they have presynaptic specializations, contain vesicles, and have a cytoskeleton similar to that of axons (Hall et al., 1989). De novo axons that emerge from the distal dendrites of cat motoneurons also have a cytoskeletal ultrastructure typical of axons (Rose et al., 2001) and are myelinated (Linda et al., 1992; Rose et al., 2001). At short post-axotomy times (i.e., 2, 4, and 8–12 weeks), nearly all de novo axons that emerge from the distal dendrites of cat motoneurons are immunoreactive for the axonal growth-associated protein-43 (GAP-43) at their distal tips (MacDermid et al., 2004); however, at all times post axotomy (i.e., 2, 4, 8–12, 20, and 35 weeks), they have no detectable immunoreactivity for the dendritic microtubule-associated protein 2a/b (MAP2a/b; MacDermid et al., 2004). Although the cellular mechanisms responsible for the formation of de novo axons from distal dendrites have not been elucidated, it is known that these processes are much more common following proximal compared with distal axotomies (Hall and Cohen, 1983, 1988b; Rose et al., 2004).

In the mammalian nervous system, recent studies have shown that intact spinal interneurons that circumvent a spinal lesion are capable of receiving new inputs from cut axons and of forming new contacts with neurons beyond the lesion (Bareyre et al., 2004; Vavrek et al., 2006). Plasticity of spinal interneurons has also been shown to play a role in autonomic dysreflexia (Schramm, 2006) and central pattern generation (Rossignol, 2006) following spinal cord injury. These studies highlight both the maladaptive and adaptive roles, respectively, of intact spinal interneurons following spinal cord injury. However, the contribution, if any, of injured spinal interneurons to the reorganization of spinal circuits is less well known. It is known that spinal interneurons, just outside the penumbrae caused by a spinal cord contusion, survive (Li et al., 1996; Liu et al., 1997). It is likely that some of these cells are proximally axotomized and thus meet the criteria for the development of de novo axons from distal dendrites. However, none of the previous studies of de novo axons that emerged from distal dendrites in the mammalian nervous system have examined spinal interneurons.

The goal of the present study was to determine whether proximally axotomized spinal interneurons develop de novo axons from their distal dendrites following a spinal cord injury in the cat. To realize this goal, we developed a novel model of spinal cord injury: a midsagittal transection of C3. This injury is specifically designed to axotomize spinal interneurons whose axons cross the dorsal or ventral commissures. These commissural interneurons (CINs) include spinothalamic, spinocerebellar, and local interneurons (Matsushita et al., 1979; Comans and Snow, 1981; Verburch et al., 1989; Sugiuchi et al., 1992, 1995; Mouton et al., 2005). The latter group has extensive projections to the

contralateral ventral horn and forms mono-synaptic connections on neck motoneurons. Due to the short distance between the commissures and the somata of CINs, the axotomy is typically 0.5–3.0 mm from the somata of the axotomized CINs. Hence, a key condition for the formation of de novo axons from distal dendrites, namely, an axotomy close to the soma, is satisfied.

Our results demonstrate that some CINs that were proximally axotomized developed de novo axons from distal dendrites. Unexpectedly, a second set of CINs that were proximally axotomized had very long processes that projected directly from the soma or from proximal dendrites immediately adjacent to the soma. We called these processes long axon-like processes (L-ALPs) because of their structural and molecular similarities to axons. Some of these processes crossed through the lesion site to the contralateral spinal cord. Preliminary descriptions of these data have been published previously (Fenrich et al., 2004).

MATERIALS AND METHODS

Midsagittal lesion of the ventral commissure at C3

All experimental protocols were approved by the Queen's University Animal Care Committee and were consistent with guidelines established by the Canadian Council of Animal Care. Specially modified scalpel blades were used for the lesions. Breakable razor blades (Fine Science Tools, North Vancouver, BC, Canada) were broken to be approximately 1 cm in length. With the aid of a dissecting microscope, the broken edge was sharpened by hand to the shape shown in Figure 1. Experiments were performed on 12 adult cats weighing between 2.5 and 4.5 kg.

The animals were anesthetized with either sodium pentobarbital (35 mg/kg i.p.) or ketamine (6.25 mg/kg i.v.) and midazolam (0.31 mg/kg i.v.) and maintained with either sodium pentobarbital (5 mg/kg i.v.) or isoflurane (1–2%), respectively. Body temperature was maintained at 37°C with a Bair Hugger (Benson Medical, Markham, ON, Canada). A dorsal laminectomy was performed to expose ~10 mm of the C3 spinal cord. The animals were secured in a stereotaxic frame, and the C3 nerves innervating the right and left biventer cervicis and complexus muscles (BCCM) were isolated and mounted on stimulating electrodes. The locations of field potentials evoked by antidromic stimulation of BCCM motoneurons were determined for both the left and right motoneuron pools. The midpoint between the field potentials was used to delineate the midline of the spinal cord. The specially modified scalpel blade was mounted on a stepping motor microdrive and aligned with a predetermined anatomical marker on the midline. The pia matter along the midline was then removed. All lesions started caudally.

Figure 1 shows the blade movements used to transect the spinal cord. The scalpel was lowered 2.5 mm into the spinal cord and then withdrawn to the surface. The blade was moved rostrally 0.75 mm and again lowered to 2.5 mm. This “saw-tooth” motion was repeated for the total length of the lesion (3–4 mm). Once the blade had been lowered to 2.5 mm at the rostral end of the lesion, the blade was moved caudally and then rostrally the entire length of the lesion several times. The identical saw-tooth action was then repeated, except that the blade was lowered to the 5-mm mark on the blade and retracted to 2.5 mm

from the dorsal surface of the cord. Again, once the blade had been lowered to the desired depth at the rostral end of the lesion, the blade was moved caudally and rostrally the entire length of the lesion several times and removed from the spinal cord at the caudal end of the lesion. A lesion depth of 5 mm was chosen. At this depth, the blade would not reach the anterior spinal artery (the dorsal-ventral extent of the C3 spinal cord of the adult cat is approximately 6 mm) and would be deep enough to transect the commissures (the tips of the ventral horns are approximately 4 mm from the dorsal surface of the cord). Based on the distribution of cervical CINs (Bolton et al., 1991; Sugiuchi et al., 1995), we estimated that CINs were axotomized at 0.5–3.0 mm from their soma.

The animals were divided into three groups. One group was used to test the parameters of the lesion ($n = 3$; see following section). These experiments were performed with the animals maintained under anesthesia and terminated several hours following the lesion; they are therefore called the non-recovery experiments. The second group of animals had survival times of 10 days ($n = 2$) or 8 weeks ($n = 2$). The third group of animals had survival times of 4–5 weeks ($n = 3$). For all the survival experiments, sutures were placed along the midline to rejoin dorsal neck muscles and close the skin incision. Postoperative pain was controlled by using hydromorphone (0.05–0.1 mg/kg SQ, every 6 hours or as required) and Metacam (Boehringer Ingelheim, Burlington, ON, Canada, 1 drop/day as required). Spastic paralysis of all four limbs occurred in two experiments soon after recovery and was invariably caused by vascular injuries that led to large spinal cavitations. These experiments were terminated very soon after the onset of paralysis, and the animals were excluded from the present study. Otherwise, the surviving animals displayed only minor and transient neurological deficits, such as hind leg ataxia. There were no noticeable changes in head movements as a result of the injury, or in the subsequent survival period. For the 10-day to 8-week survival experiments, the animals were anesthetized with sodium pentobarbital anesthesia (same anesthesia protocol as described above), and the spinal cord was perfused and fixed (see Perfusion and fixation section below).

Staining of neurons with extracellular injections of Neurobiotin

For all non-recovery experiments and 4–5-week survival experiments, a laminectomy was performed to expose the spinal cord from C1 to C5. The nerves innervating the trapezius (TRAP) muscles, the C2 and C3 nerves innervating the splenius (SP) muscles, and the C2, C3, and C4 nerves innervating BCCM were isolated and mounted on stimulating electrodes. For the 4–5-week survival experiments, tissue that had accumulated above the surface of the injured spinal cord was cut away by using curved spring scissors (Fine Science Tools). All animals were paralyzed with gallamine triethiodide (Sigma, 2.5–5 mg/kg/hr i.v.) and ventilated by using a respirator.

Spinal neurons were extracellularly stained with iontophoretic injections of 12% Neurobiotin (Vector, Burlington, ON, Canada) in 0.5 M KCl and 0.1 M Tris-HCl buffer, pH 8.2. The glass pipettes were broken to achieve a tip diameter of 4 μ m. Most injections were made on electrode tracks that traversed both SP and BCCM antidromic field potentials; the former was used as a landmark for the medial-lateral midpoint of the ventral horn, and the

latter served as an index of the most ventral extent of the ventral horn (cf. Richmond et al., 1978). All injections were made into laminae VII/VIII/IX.

In non-recovery experiments, two injections were made in each electrode track, one 200 μm dorsal to the BCCM field potential, and another 800 μm dorsal to the BCCM field potential. Injections were made with 1–5- μA positive pulses for 90 seconds with a 10-second on/off duty cycle (Midgard Electronics, Canton, MA, USA, model CS3). Each injection track was separated by 1 mm rostrally and caudally, and injections were made unilaterally throughout the entire rostral-caudal extent of each lesion and for several millimeters rostral and caudal of each lesion.

In the 4–5-week survival experiments, injections were made with 1- μA positive pulses for 1 minute with a 10-second on/off duty cycle. Each injection stained approximately 2–10 neurons. One or two injections were made in each track. The single injection was made 600–700 μm dorsal to the BCCM antidromic field potential, and the double injections were made at the location of the BCCM antidromic field potential and 800 μm dorsal to the first injection. Injections were separated by 2 mm rostrally and caudally, and injections were made bilaterally throughout the entire rostral-caudal extent of the lesion.

Perfusion and fixation

Following an injection of heparin (25,000 IU), all animals were euthanized with an overdose of sodium pentobarbital. The animals were perfused with saline (1 liter), followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 30 minutes. The spinal cord was excised, postfixed overnight, and subsequently transferred to 15% sucrose in 0.1 M sodium phosphate buffer (NaPBS) for several days prior to histological processing.

Immunohistochemistry and visualization of Neurobiotin

Tissue was cut with a freezing microtome (Leitz) into serial sections that were 50 μm thick in the horizontal plane. The sections were incubated in 1% sodium borohydride in NaPBS for 1 hour. Non-specific labeling was blocked by incubating the sections overnight in 10% normal goat serum and 0.3% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) in 0.02 M potassium phosphate buffered saline (KPBS), pH 7.4. The sections were initially processed with mouse monoclonal antibodies raised against partially purified neonatal rat GAP-43 protein (1:40,000; clone 9-1E12; gift of David Schreyer, University of Saskatchewan). The GAP-43 antibodies were visualized with Cy3-conjugated secondary antibodies (1:100, host goat; Jackson Laboratories, Mississauga, ON, Canada). Western blots were performed as follows: Feline spinal cord was homogenized on ice with a Dounce glass homogenizer in TPEN protein extraction reagent (Pierce, Rockford, IL) containing 10% Nonidet P-40, 1% APS, and protease and phosphatase inhibitor cocktails (Sigma). Protein samples (10 μg for GAP-43 and 5 μg for MAP2a/b) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% Tris-glycerin acrylamide gel (GAP-43 analysis) or a 4–10% gradient Tris-glycine acrylamide gel (MAP2a/b analysis) and were transferred with CAPS buffer (pH 11; Sigma) to PVDF membranes (Immobilon-P, 0.45 μm , Millipore, Billerica, MA). Membranes were probed with GAP-43 antibodies (1:5,000) or MAP2a/b antibodies (1:5,000), respectively, and then with horseradish peroxidase (HRP)-conjugated

secondary antibodies (1:5,000, host donkey; Amersham, Oakville, ON, Canada). The GAP-43 antibodies stained a single protein band of ~53 kDa on Western blots of feline spinal cord (Fig. 1C). Staining of the injured spinal cord produced a pattern of somatic GAP-43 immunoreactivity that was identical to that of GAP-43-immunoreactive somata previously described in feline retina (Coblentz et al., 2003) and rat spinal cord (Weaver et al., 1997). To visualize Neurobiotin, the sections were processed with streptavidin conjugated to Alexa 488 (1:100; Molecular Probes, Hornby, ON, Canada). Sections were mounted on subbed slides with Vectashield mounting medium (Vector) and stored at 2–4 °C.

In the 4–5-week survival experiments, the tissue sections were reprocessed to visualize MAP2a/b immunoreactivity. Sections were reprocessed with mouse monoclonal antibodies raised against bovine brain microtubule protein (1:10,000; clone AP20; Chemicon, Temecula, CA, cat. no. MAB3418). The MAP2a/b antibodies were visualized with Cy3-conjugated secondary antibodies (1:100, host goat; Jackson Laboratories). These antibodies stain a single band of high molecular weight on Western blots of feline spinal tissue (Fig. 2D). Staining of the injured spinal cord produced a pattern of strong dendritic and weak or absent somatic MAP2a/b immunoreactivity that was identical with previous descriptions of MAP2 staining in adult rat cortical neurons (Bury and Jones, 2002) and also produced a patterns of strong dendritic immunoreactivity that was identical to previous descriptions in cat motoneurons (MacDermid et al., 2002). After this reprocessing, former GAP-43 labeling with Cy3 could not be detected. Because this procedure also reduced the intensity of Neurobiotin labeling, the tissue was also reprocessed with streptavidin conjugated to Alexa 488 (1:200; Molecular Probes).

Reconstruction techniques

Neurons were reconstructed by using a fluorescent microscope (Olympus BX60) equipped with 40× (0.75 NA) and 60× (0.90 NA) dry immersion lenses and a Neurolucida System (MicroBrightfield, Williston, VT, USA) (v5.05.4). A computer-based reconstruction of a neuron is a three-dimensional tracing of a neuron in which each process is represented as a series of connected points starting at a point of origin. All processes were followed to their termination, defined by an abrupt loss of Neurobiotin, or the point at which Neurobiotin staining became too faint. The latter “endings” were labeled as “incomplete.”

Despite our efforts to limit the number of neurons stained at each injection site, on several occasions complex networks of processes were stained that could not be accurately reconstructed by using a standard fluorescent microscope. To resolve the paths taken by some processes in these networks, images of these regions were captured by using a confocal microscope (Leica TCS SP2 multi photon). The process of interest was followed through each confocal optical slice and incorporated into the reconstruction. For cases in which a process entered an injection epicenter, the reconstruction was stopped at the injection site, and the “ending” was marked as “incomplete.”

Identification of double-labeled processes

Alexa 488 and Cy3 were visualized by using band-pass excitation and emission filters (Chroma Technology, Rockingham, VT, USA) that were designed to minimize the risk of

visualizing Cy3 with the Alexa 488 filter set and vice versa. The distribution of GAP-43 or MAP2a/b immunoreactivity was mapped during the reconstruction process. Markers were assigned to indicate the start and end of double labeling and were attached to the reconstruction to map the distribution of GAP-43 and MAP2a/b immunoreactivity. When double labeling was difficult to determine by simple visual inspection, digital images were taken of each fluorochrome by using a color CCD camera (DC330, Dage-MTI, Michigan City, IL) and integrating software from Image Pro Plus (Media Cybernetics v4.5, Silver Springs, MD). Line-profile histograms of the process were acquired by using Image Pro Plus, and the relative intensity of the Cy3 labeling of the process was compared with background intensity. If the intensity of the process was at least 1.5-fold the intensity of the background intensity, the process was placed in the double-labeled category; otherwise the process was considered to be single labeled. In rare cases in which this method failed to distinguish unequivocally between double and single labeling, the processes were classified as “unknown.”

RESULTS

Extent and reliability of the lesion: results from the non-recovery experiments

Rostral-caudal distribution of Neurobiotin-stained processes that crossed the midline—To determine whether the lesion protocol caused a complete transection of the commissures, unilateral Neurobiotin injections were made immediately after completing the lesion(s) in three experiments. These experiments were terminated several hours later. We examined a total of five lesions. Two experiments had a single lesion at C3, whereas one experiment had three separate lesions: one in C2, one in C3, and one in C4. Figure 2A shows the distribution of Neurobiotin-labeled processes with medial-lateral projections near the midline for one lesion. These processes were uniformly distributed medial to the injection sites, but some ended abruptly as they approached the midline. In contrast, other processes formed a dense network that crossed the midline. These processes were located rostrally and caudally to those that failed to cross the midline, and this distribution revealed a distinct and continuous region along the midline in which Neurobiotin-stained processes were absent. The same pattern of Neurobiotin labeling was seen in the region of all five lesions and suggests that the distribution of Neurobiotin processes that fail to cross the midline is a reliable means of identifying the extent of the lesion. These results also indicate that our injury protocol leads to a complete transection of the commissures.

However, there is a caveat to this conclusion. Despite the rostral-caudal proximity of the injections of Neurobiotin, there were regions between each injection where the labeling of Neurobiotin processes was sparse. Hence, we cannot exclude the possibility that the absence of Neurobiotin processes in some regions along the midline was due to a failure to stain all processes that crossed the midline. To address this issue, we used a second approach to analyze the distribution of processes that crossed the midline.

Rostral-caudal distribution of dendrites along the midline—To verify that all processes were cut within the midline zones in which Neurobiotin-stained processes were absent, we determined the distribution of dendrites that crossed the midline in the vicinity of

the lesion. MAP2a/b immunoreactivity was used as a marker for dendrites (Tucker, 1990; MacDermid et al., 2002). As shown in Figure 2B–F, MAP2a/b labeling along the midline abruptly decreased at the rostral border of the zone where Neurobiotin-stained processes were absent. Within this zone, MAP2a/b labeling was either absent or restricted to small regions at the rostral or caudal boundaries (e.g., Fig. 2E). These experiments indicate that the zone in which Neurobiotin-stained processes were absent is an accurate measure of the extent of the lesion. Moreover, the distribution of MAP2a/b immunoreactivity along the midline suggests that it can be used as an alternative means of defining the location of the lesion.

GAP-43-immunoreactive somata and processes

Frequency and distribution of GAP-43-immunoreactive somata—Following postlesion periods of 10 days or 8 weeks, many GAP-43-immunoreactive neurons were found in C3. The perinuclear region and proximal dendrites of these neurons were well labeled with Cy3 (Fig. 3A,B). In contrast, the nucleus was weakly stained and appeared as a dark core in the center of the soma. As summarized in Table 1, the number of GAP-43-immunoreactive somata was much greater at 8 weeks post lesion than at 10 days post lesion.

These results are consistent with previous studies demonstrating that many neurons express GAP-43 following axotomy (Schwab and Bartholdi, 1996; Dusart et al., 2005). To verify that axotomy alone (i.e., not other factors related to the trauma caused by the lesion) was responsible for the expression of GAP-43 in our model of spinal cord injury, we determined the distribution of GAP-43-immunoreactive somata relative to the location of the lesion and compared the distribution with the known paths taken by axons of CINs in C3.

In the transverse plane, the axons of CINs project medially, cross the midline, and ascend or descend in the spinal cord in the ventral-medial, lateral and dorsal-lateral funiculi (Sugiuchi et al., 1992, 1995; Matsuyama et al., 2004, 2006). In keeping with this path and the causal link between the expression of GAP-43 in CINs and transection of their axons, GAP-43-immunoreactive neurons were only found on the left side of the spinal cord in one experiment in which the lesion traversed the left ventral horn without transecting the commissures (Fig. 3C). In contrast, GAP-43-immunoreactive neurons were found on both sides of the spinal cord following a lesion that transected CIN axons bilaterally by a lesion through the commissures at the midline (Fig. 3D). As expected, many of these somata were found in regions that correspond to the location of local commissural interneurons (Sugiuchi et al., 1992, 1995), as well as spinothalamic and spinocerebellar neurons (Matsushita et al., 1979; Comans and Snow, 1981; Verburgh et al., 1989; Mouton et al., 2005).

The rostral-caudal distribution of GAP-43-immunoreactive somata was also consistent with the known trajectory of axons from CINs. When they are viewed in the horizontal plane, the axons of local commissural interneurons, spinothalamic neurons, and central cervical nucleus neurons follow a path to the commissure that is perpendicular to the rostral-caudal axis of the spinal cord (Wiksten, 1979; Comans and Snow, 1981; Sugiuchi et al., 1992, 1995). Thus, only CINs that are at, or close to, the same rostral-caudal level of the lesion will be axotomized. Figure 3E shows the location of GAP-43-immunoreactive somata in the horizontal plane from an 8-week experiment. The vast majority of these cells were located at

the same rostral-caudal level as the lesion that transected the commissures. This distribution pattern was confirmed quantitatively by comparing the cumulative frequency of GAP-43-immunoreactive somata with the rostral-caudal distance spanned by the lesion (Fig. 3F). For all four animals examined, there was an abrupt increase in the cumulative frequency of GAP-43-immunoreactive somata at the rostral border of the lesion and an equally abrupt plateau at the caudal border of the lesion. These results, together with the distribution of GAP-43-immunoreactive somata in the transverse plane, lead to the conclusion that the somatic expression of GAP-43 is a reliable means of identifying axotomized CINs.

Frequency and distribution of GAP-43-immunoreactive processes—Many de novo axons that emerged from distal dendrites of axotomized motoneurons had GAP-43-immunoreactive tips (Rose et al., 2001; MacDermid et al., 2002, 2004). When these GAP-43-immunoreactive processes were examined in isolation (i.e., in the absence of intracellular staining of axotomized motoneurons), the GAP-43 immunoreactivity was confined to two types of processes: simple cylindrical branches or more elaborate bush-like arbores with several short and bulbous branches (Rose et al., 2001). These processes matched the structure of the distal regions of de novo axons that emerged from distal dendrites of motoneurons that were visualized after intracellular staining (Rose et al., 2001). Regardless of their morphology, all processes that were GAP-43 immunoreactive were confined to regions of the ventral horn that contain the dendrites of axotomized motoneurons (Rose et al., 2001). We also found GAP-43-immunoreactive processes in the present experiments. These processes were classified as simple, as defined by no more than one swelling or bifurcation (Fig. 4A); or complex, as defined by two or more bifurcations or swellings (Fig. 4B,C). As summarized in Table 1, at 10 days post lesion, simple processes outnumbered complex processes, although the exact ratio varied from experiment to experiment. At this time-point, complex processes ranged in morphology from a small network of very short bulbous branches to larger and more complex arbores with many irregular swellings (Fig. 4B). By 8 weeks post lesion, the average number of GAP-43-immunoreactive processes had doubled, but the ratio of simple to complex processes remained similar (Table 1). At this time-point, many of the complex processes consisted of a single branch with numerous spherical swellings (Fig. 4C). The structure of the simple processes was unchanged.

Figure 4D,E shows the location of GAP-43-immunoreactive processes in the horizontal plane from the same experiment shown in Figure 3E. Most complex processes were found in the gray matter at the same rostral-caudal level as the lesion (Fig. 4D). In contrast, GAP-43-immunoreactive processes that were classified as simple were more widely distributed (Fig. 4E). Many were found more than 3 mm rostral and caudal to the lesion. These results suggest that many, but not all, GAP-43-immunoreactive processes are located in the vicinity of axotomized CINs. To address this relationship more directly, we compared the cumulative frequencies of GAP-43-immunoreactive processes and somata with the rostral-caudal distance spanned by the lesion (Fig. 4F–I). In each experiment there was a very close correspondence between the rostral-caudal distribution of complex GAP-43-immunoreactive processes and GAP-43-immunoreactive somata. The frequency of simple GAP-43-immunoreactive processes was also higher in the vicinity of the axotomized CINs at 8 weeks

post injury (Fig. 4H,I and Table 1), but at 10 days post injury (Fig. 4F,G and Table 1) these processes were uniformly distributed throughout the rostral-caudal extent of the tissue examined. In all experiments, at least 46% of the simple GAP-43-immunoreactive processes were located outside the rostral-caudal region occupied by axotomized CINs.

The results of these experiments satisfy three critical criteria that are implicit in the hypothesis that axotomized CINs develop *de novo* axons from distal dendrites following a spinal cord injury. First, GAP-43-immunoreactive processes were present after a spinal cord injury that axotomized CINs. Second, the morphology of many of these processes was very similar to the GAP-43-immunoreactive regions seen in *de novo* axons that emerged from the distal dendrites of axotomized motoneurons. Finally, many of these processes, especially those classified as complex, were located in regions occupied by the dendritic trees of CINs. However, none of these results directly demonstrate that these GAP-43-immunoreactive processes arose from the dendrites of axotomized CINs, nor do they take into account that *de novo* axons that emerged from distal dendrites of motoneurons at long post-axotomy intervals did not have GAP-43-immunoreactive tips. To address this issue, we took advantage of the somatic expression of GAP-43 as a means of identifying axotomized CINs in combination with extracellular staining techniques to follow the trajectory of dendrites from their origin on the somata of axotomized CINs to their terminations.

Reconstructions of axotomized CINs

Ten CINs were partially or completely reconstructed. All 10 reconstructed cells were selected from a population of cells stained in three experiments following a 4–5-week survival period. This post-axotomy period was chosen because animals at this time point had the greatest number of GAP-43-immunoreactive somata: 864, 1,092, and 1,191 somata per experiment. The selection criteria for reconstruction of a cell included: the presence of a GAP-43-immunoreactive soma (identification criterion for axotomized CINs; see above), being well stained by Neurobiotin, and being located in regions with little overlap with other Neurobiotin-stained cells. The last criterion was designed to minimize the risk of “losing” processes in areas of dense networks of Neurobiotin processes. Despite this criterion, some processes on several cells were followed into zones containing many Neurobiotin processes and could not be followed to their termination. To address this problem, only processes that fulfilled one of the following two criteria were included in the reconstructions: 1) the process could be followed to a well-defined and abrupt termination in staining; or 2) the process gradually disappeared as it was followed distally. The latter type of process was typically found several millimeters from the soma and is identified in all reconstructions. The ends of these processes were assigned a unique marker.

MacDermid et al. (2002, 2004) described three key features of the distribution of GAP-43 and MAP2a/b immunoreactivity in *de novo* axons arising from distal dendrites of motoneurons: 1) although all GAP-43-immunoreactive processes that emerged from distal dendrites were *de novo* axons, not all *de novo* axons that emerged from distal dendrites were GAP-43-immunoreactive, especially at longer axotomy intervals (i.e., 20 and 35 weeks); 2) the absence of MAP2a/b immunoreactivity was a feature of all *de novo* axons that emerged from distal dendrites, regardless of the post-axotomy interval; and 3) GAP-43

immunoreactivity was restricted to the distal tips of de novo axons that emerged from distal dendrites, whereas MAP2a/b immunoreactivity was absent throughout their length. Therefore, for this study, de novo axons that emerged from distal dendrites were identified as any process that emerged from a distal dendrite and lacked MAP2a/b immunoreactivity. GAP-43 immunoreactivity was used as a further indicator of de novo axons but was not used to define de novo axons, because not all de novo axons are GAP-43 immunoreactive (see above).

The tissue was first processed to visualize Neurobiotin labeling and GAP-43 immunoreactivity. The CINs were reconstructed and the distribution of GAP-43 immunoreactivity was determined. The tissue was then reprocessed to visualize MAP2a/b immunoreactivity and Neurobiotin labeling. The same 10 neurons were reconstructed a second time to describe the distribution of MAP2a/b immunoreactivity. Unexpectedly, reprocessing the tissue a second time improved the Neurobiotin labeling. As a consequence, several of the processes that could not be reconstructed to their terminations in the first reconstruction, perhaps due to fading during the original reconstructions, could be followed to their terminations following Neurobiotin reprocessing.

CINs with de novo axons that emerged from distal dendrites—As summarized in Table 2, four CINs had de novo axons that arose from their distal dendrites. The axons of two of these cells ended abruptly in the gray matter. Despite a careful search in the vicinity of the somata of the other two cells, no axon could be found. In total, we found 25 de novo axons that emerged from distal dendrites. These were traced back to 8 of the 17 primary dendrites whose higher order branches were completely reconstructed.

Four of the de novo axons that emerged from distal dendrites were GAP-43 immunoreactive (Fig. 5). Similar to de novo axons that arise from the distal dendrites of motoneurons, GAP-43 immunoreactivity was restricted to the distal tips of the de novo axons that emerged from the distal dendrites of CINs (Fig. 6A–C). MAP2a/b immunoreactivity was absent in these regions and extended into adjacent proximal segments (Fig. 6D–F), as previously described for de novo axons arising from the distal dendrites of motoneurons. Proximal dendritic segments were MAP2a/b immunoreactive (Fig. 6G–I). With one exception, the GAP-43-immunoreactive tips had a single terminal swelling; the exception had two bifurcations and a swelling. Overall, the de novo axons that emerged from the distal dendrites of CINs and had GAP-43-immunoreactive tips were simpler than the complex arborizations formed by many de novo axons arising from the distal dendrites of motoneurons.

Eighteen de novo axons that emerged from distal dendrites of CINs were not immunoreactive for GAP-43. All these processes were identified based on the absence of MAP2a/b immunoreactivity. Three other de novo axons that emerged from distal dendrites were also identified by their lack of MAP-2a/b immunoreactivity. However, the absence or presence of GAP-43 immunoreactivity in these processes could not be determined, because they were only found after reprocessing for Neurobiotin. Sixteen of these 21 de novo axons had multiple structural features typical of axons and emerged from MAP2a/b-immunoreactive dendrites. Figure 7A shows two de novo axons that arose from the same

primary dendrite. The de novo axon shown in the left inset of Figure 7A had an arboreal appearance. Like axons, many of the branches of this process emerged at right angles to their parent branches and followed a tortuous path. The de novo axon shown in the right inset had a collateral-like structure (i.e., multiple fine terminal branches) with three bifurcations and several bouton-like swellings. Confocal microscopic images of these bouton-like swellings indicated that several formed close appositions with MAP2a/b-immunoreactive dendrites (Fig. 7B–D). These appositions meet criteria that have been established to define synaptic contacts at the light microscopic level (Fyffe, 1991; Rose et al., 1999): 1) the axon formed a round or elliptical swelling whose diameter was at least twice that of the adjacent shaft; 2) there was no discernible gap between the axon and dendrite; and 3) both the swelling and the dendrite are in the same focal plane at the site of the potential contact. Four de novo axons that emerged from distal dendrites of three CINs were examined for synapse-like contacts with MAP2a/b-immunoreactive dendrites. Three of these four processes formed synapse-like contacts. These de novo axons emerged from dendrites arising from two CINs.

Two of the 21 de novo axons that emerged from distal dendrites followed a relatively straight trajectory, had diameters that were the same as the MAP2a/b immunoreactive process from which they emerged, and had less than one bifurcation and/or varicosity (Fig. 8A).

The immunocytochemical characteristics of the processes that gave rise to the last three of the 21 de novo axons that emerged from distal dendrites were unusual. Unlike all other de novo axons that arose from MAP2a/b-immunoreactive dendrites, they arose from dendrites that lacked MAP2a/b immunoreactivity. In these cases, the loss of MAP2a/b immunoreactivity could not be used to define the end of the dendrite and the start of the de novo axon. Thus, structural features typical of dendrites were used to determine the end of the dendrite. Dendrites taper and have acute angled branches (Peters et al., 1991). Therefore the point at which the proximal process ceased to have a dendritic morphology was used as the start of the de novo axon. For example, as shown in Figure 8B, the dendrites proximal to the arrowheads taper and branch at acute angles, whereas distal to the arrowheads the de novo axons have irregular diameters, large varicosities, right-angled branches, and arboreal-like structures.

CINs with long axon-like processes—As summarized in Table 2, five CINs gave rise to long axon-like processes (L-ALPs). Unlike de novo axons that emerged from distal dendrites, L-ALPs projected directly from the soma or a very proximal dendrite (i.e., within 25 μ m of the soma and prior to the first dendritic branch point). Like axons, L-ALPs lacked MAP2a/b immunoreactivity and had many morphological features typical of axons such as right-angled branches, following a tortuous path, a uniform diameter, collateral-like branching patterns, and bouton-like varicosities (Peters et al., 1991). Three L-ALPs crossed the midline, one projected to the ipsilateral spinal cord, and one had projections to both the ipsilateral and contralateral spinal cord. These processes were followed for distances of ~3,400 μ m to a maximum of ~9,700 μ m. However, because of the gradual loss of Neurobiotin staining in the most distal extremities of the L-ALPs, none could be followed to their termination. GAP-43 immunoreactivity was not detectable in L-ALPs.

Figure 9A shows an example of an L-ALP. This L-ALP followed a very tortuous trajectory that bifurcated into two main branches. One branch projected to the contralateral ventral-medial funiculus; the other entered the ipsilateral lateral funiculus. This L-ALP gave rise to two fine-diameter branches near the soma. These fine branches projected toward and across the midline (Fig. 9A, left inset) and had many right-angled branches and irregular varicosities, several of which formed close appositions with MAP2a/b-immunoreactive dendrites (data not shown). Two small-diameter branches emerged at right angles from the ipsilateral branch of the L-ALP (Fig. 9A, right inset). These small-diameter branches had bouton-like varicosities that formed synapse-like contacts with MAP2a/b-immunoreactive dendrites (Fig. 9B–D). Three of the five L-ALPs had bouton-like swellings that formed synapse-like contacts.

Figure 10 is a schematic of all five L-ALPs. Two of the L-ALPs followed courses that were unusual (i.e., ipsilateral projections). The morphology of two L-ALPs was unusual. These L-ALPs had ipsilateral collateral branches and large irregular varicosities (see Discussion). L-ALPs projected from CINs that did not have any de novo axons that emerged from distal dendrites. The absence of de novo axons from distal dendrites was based on the complete reconstruction of the dendritic trees of four of the neurons with L-ALPs and a partial reconstruction of the dendritic tree of the remaining CIN.

CINs with no de novo axons that emerged from distal dendrites or L-ALPs—As summarized in Table 2, one neuron had no de novo axons that emerged from distal dendrites or L-ALPs. However, the reconstruction of the dendritic tree of this CIN was limited to only two primary dendrites and their branches as the other primary dendrites projected into the injection epicenter (see Materials and Methods).

L-ALPs within the lesion site

Four of the five L-ALPs projected to the contralateral spinal cord. This result raises the question: were these processes within regions of the spinal cord that were transected by the lesion? To answer this question, we compared the rostral-caudal positions at which the L-ALPs crossed to the contralateral spinal cord with the distribution of dendrites that crossed the midline. As shown in the non-recovery experiments, the absence of MAP2a/b immunoreactivity along the midline provides an accurate measure of the extent of the lesion immediately following a lesion. The black bars in Figure 11A and B indicate the frequency of MAP2a/b-immunoreactive dendrites that crossed the midline for the two experiments in which midline-crossing L-ALPs were found. The distributions of MAP2a/b immunoreactivity along the midline at 4–5 weeks post axotomy are consistent with the distribution of MAP2a/b immunoreactivity immediately following a lesion and with the distribution of GAP-43-immunoreactive somata (see below). This indicates that MAP2a/b immunoreactivity along the midline remains a valid means of defining the extent of the lesion at longer post-axotomy times. All four L-ALPs crossed the midline at points where MAP2a/b immunoreactivity was absent, suggesting that the L-ALPs were within regions of the midline transected by the lesion.

To confirm further that the L-ALPs were within regions that were transected by the lesion, the points at which the L-ALPs crossed the midline were compared with the distribution of GAP-43-immunoreactive somata. As previously described, the distribution of GAP-43-immunoreactive somata is equivalent to the distribution of axotomized CINs. Because the axons of intact CINs project to the contralateral cord at nearly the same rostral-caudal level as their somata (Wiksten, 1979; Comans and Snow, 1981; Sugiuchi et al., 1992, 1995), the rostral-caudal distribution of GAP-43-immunoreactive somata is another indicator of the rostral-caudal extent of the lesion. As shown in Figure 11C and D, the rostral-caudal location of the GAP-43-immunoreactive somata in these experiments formed a discrete zone with abrupt rostral and caudal borders. All the L-ALPs crossed to the contralateral cord at rostral-caudal levels that were well within the rostral-caudal zone occupied by GAP-43-immunoreactive somata. These results confirm the results based on the distribution of MAP2a/b immunoreactivity at the mid-line and suggest that L-ALPs were within zones that were transected by the lesion.

DISCUSSION

This study is the first to show that mammalian spinal commissural interneurons (CINs) develop *de novo* axons from distal dendrites following a spinal lesion. *De novo* axons from distal dendrites of CINs had morphological and molecular features typical of axons and resembled *de novo* axons that originate from the distal dendrites of permanently axotomized motoneurons and autonomic neurons (MacDermid et al., 2004; Hoang et al., 2005). By using confocal microscopy, we also showed that *de novo* axons that emerged from the distal dendrites of CINs form synapse-like contacts with MAP2a/b-immunoreactive dendrites.

The second principal finding was unexpected. Some axotomized CINs had long axon-like processes (L-ALPs). These processes were unexpected because they followed a trajectory through the injury site or they projected to funiculi that were inconsistent with the known path taken by axons of CINs (i.e., the ipsilateral lateral funiculus instead of contralateral funiculi). In contrast to *de novo* axons that emerged from distal dendrites, L-ALPs projected from the soma or very proximal dendrites.

De novo axons emerging from distal dendrites

Previous studies used several methods to distinguish axotomized neurons from intact neurons. These methods included identification based on morphological features that were specific to the axotomized cells (Hall and Cohen, 1983, 1988b; Hall et al., 1989), intracellular staining of neurons in regions known to contain the axotomized cells and visualization of the transected axon (Linda et al., 1985, 1992), antidromic stimulation of the proximal stump of the cut nerve (Rose and Odlozinski, 1998; Rose et al., 2001; MacDermid et al., 2002, 2004), or identification based on neurochemical markers that were unique to the class of neurons that were axotomized (Hoang et al., 2005). None of these methods were suitable for the identification of axotomized CINs. In the present study, antidromic identification of the neurons was not feasible due to the loss of the axons, neurochemical identification was not possible because the neurochemical features of CINs are not known, and location and morphology, together, are not a definitive means of identifying CINs. As a

consequence, we identified axotomized CINs based on the presence of GAP-43 immunoreactivity in the soma. This approach has been used previously by Doster et al. (1991), who showed that only axotomized retinal ganglion cells express GAP-43, whereas intact retinal ganglion cells immediately adjacent to an injury do not express GAP-43. In the present study, the non-recovery experiments confirmed that the somatic expression of GAP-43 is also a rigorous means of distinguishing intact CINs from axotomized CINs.

Most studies have used intracellular staining techniques to visualize de novo axons arising from distal dendrites (Hall and Cohen, 1983, 1988b; Linda et al., 1985; Hall et al., 1989; Linda et al., 1992; Rose and Odlozinski, 1998; Rose et al., 2001; MacDermid et al., 2002, 2004). In this study, we stained neurons with extracellular iontophoretic injections of Neurobiotin rather than using intracellular staining techniques. This staining technique sometimes resulted in staining of multiple nearby cells and high background at the injection epicenter. This rendered the reconstruction of entire neurons more difficult and sometimes not possible. In the absence of complete reconstructions, this approach was not ideally suited to determine the frequency of neurons with de novo axons that emerged from distal dendrites or the number of de novo axons that emerged from distal dendrites on a cell-by-cell basis. However, despite these limitations, it was possible to follow many dendrites and de novo axons that emerged from distal dendrites to their terminations by using computer-aided reconstruction techniques. Also, although this approach was not ideally suited for collecting data from a large number of cells, extracellular Neurobiotin injections appeared to stain cells indiscriminately. Therefore the 10 reconstructed neurons are a randomly selected population of axotomized CINs from laminae VII, VIII, and IX, which indicates that the reconstructed neurons are, most likely, a representative sample of axotomized CINs.

An absence of MAP2a/b immunoreactivity was the main criteria used to identify de novo axons that emerged from distal dendrites. The validity of this criterion rests on the assumption that dendrites always contain MAP2a/b. This is certainly the case for intact neurons (Tucker, 1990; MacDermid et al., 2002). However, MAP2a/b immunoreactivity is lost from distal dendrites in intact neurons several millimeters from transection injuries (Meehan et al., 2003). One might interpret these data as invalidating the absence of MAP2a/b immunoreactivity as an indicator for de novo axons that emerged from distal dendrites. However, other data from the present study speak against this possibility. MAP2a/b immunoreactivity was present throughout the dendritic trees of CINs with L-ALPs. This indicates that the loss of MAP2a/b immunoreactivity from distal dendrites cannot be simply attributed to the fact that these cells were in the vicinity of a lesion. Furthermore, nearly all de novo axons that emerged from distal dendrites and lacked MAP2a/b immunoreactivity also had morphological features typical of axons rather than dendrites. In contrast, the dendrites without MAP2a/b immunolabeling described by Meehan et al. (2003) were not deemed to meet morphological criteria typical of de novo axons that emerge from distal dendrites (Meehan, personal communication). Therefore, in the present study, although we cannot fully discount the possibility that MAP2a/b was lost from some distal dendrites of CINs for reasons other than the formation of de novo axons, there is strong evidence indicating that a lack of MAP2a/b immunoreactivity is a reliable marker for de novo axons emerging from distal dendrites of CINs.

Similarities and differences between de novo axons that emerged from the distal dendrites of CINs and motoneurons—The de novo axons that emerged from the distal dendrites of CINs share morphological features with de novo axons that emerged from the distal dendrites of cat motoneurons at 20 and 35 weeks post axotomy. For instance, the de novo axons that emerged from distal dendrites of both types of neurons had right-angled branches, collateral-like branching patterns, and bouton-like swellings (MacDermid et al., 2004). De novo axons emerging from distal dendrites of motoneurons at earlier post-axotomy intervals did not have these features. Because all of the de novo axons emerging from distal dendrites of CINs were observed at 4–5 weeks post injury, this suggests that de novo axons that emerge from the distal dendrites of CINs develop faster. This suggestion is consistent with the low frequency of GAP-43-immunoreactive de novo axons emerging from dendrites of CINs (4/25) 4–5 weeks post injury. In motoneurons, most (106/118) de novo axons from distal dendrites are GAP-43 immunoreactive until 20–35 weeks post axotomy, at which point the frequency decreases to zero (MacDermid et al., 2004). It is not clear why de novo axons appear to develop faster from the distal dendrites of CINs compared with motoneurons, but it may be related to the proximity of the axotomy (3 mm versus 15–25 mm) or fundamental differences in the cells themselves.

Approximately one-half of the axotomized CINs sampled developed de novo axons from their distal dendrites. In contrast, all proximally axotomized motoneurons appear to develop de novo axons from their distal dendrites (cf. MacDermid et al., 2004). Those CINs with de novo axons had an obvious axonal stump or lacked a discernible axon (presumably due to axonal retraction; see Zhang et al., 2005; Kerschensteiner et al., 2005). In contrast, five of the six CINs without de novo axons emerging from their distal dendrites had L-ALPs. Thus, the emergence of de novo axons from the distal dendrites of CINs appears to be conditional on the absence of an L-ALP.

L-ALPs

Four of the five L-ALPs crossed the midline at the same rostral-caudal location as the lesion. This trajectory suggests that transected axons of CINs have an intrinsic ability to regenerate through a spinal lesion. However, there is recent acceptance of evidence that transection injuries may fail to transect all the axons at the site of the lesion (Steward et al., 2003). Thus, it is possible that these L-ALPs are spared axons. Steward et al. (2003) put forth seven criteria to distinguish regenerated from spared axons. To determine whether L-ALPs are regenerating axons or spared axons, our data are discussed in the context of these criteria.

Criterion I: the axon extends into the injury site—This criterion is based on the expectation that 100% of the axons in the injury site were transected. In a series of non-recovery experiments, we made multiple injections of Neurobiotin into the ventral horn. These injections spanned a zone that extended beyond the rostral and caudal extent of the lesion. Within the regions of the lesions, we were unable to find Neurobiotin-labeled processes that crossed the midline despite a thorough examination of all serial sections that contained the commissures. These results suggest that the crossing processes stained by Neurobiotin were transected by the lesion. However, not all processes that cross the midline were stained in these experiments, nor can we exclude the possibility that the absence of

Neurobiotin staining opposite the lesion was due to a functional disruption of Neurobiotin transport. Dendritic staining based on antibodies to MAP2a/b is not subject to either of these caveats. Thus, the very close match between the midline regions lacking MAP2a/b-immunoreactive processes and Neurobiotin staining provides a more persuasive case for the conclusion that all commissural axons were transected. Based on these criteria, all four L-ALPs that crossed the midline were not spared, because all crossed the midline through regions that were lacking MAP2a/b immunoreactivity. However, this conclusion ignores the possibility that the loss of MAP2a/b staining may be due to an absence of MAP2a/b, rather than a loss of the dendrites themselves (cf. Zhang et al., 2000). Thus, despite the congruence of the Neurobiotin and MAP2a/b data, it is impossible to claim that 100% of the commissural axons were transected by the lesion. It was for this reason that we only examined neurons whose somata were immunoreactive for GAP-43. Because the expression of GAP-43 appears to be contingent on axotomy (Doster et al., 1991; present data), we used this feature as another means of confining our study to axotomized cells. Thus, on balance, the data relevant to criterion I provide strong support against the possibility that at least four L-ALPs were spared axons.

Criterion II: the axon extends into a graft or transplant—This criterion is not applicable to this study because there were no grafts or transplants.

Criterion III: the axon originates at or near the site of injury—All the L-ALPs were reconstructed based on serial sections. This allowed us to trace these processes proximal, and distal, to the lesion site. Thus four of the five L-ALPs met criterion III.

Criterion IV: the axon takes an unusual course through the uninjured CNS—Two of five L-ALPs projected to the ipsilateral white matter. Given that CINs are defined by a contralateral axonal projection, the path of these L-ALPs is very unusual. However, two other L-ALPs followed a path to the contralateral ventromedial funiculus that is indistinguishable from the path taken by axons of intact CINs (cf. Fig. 10 with Sugiuchi et al., 1992, 1995). Thus, as a group, L-ALPs do not consistently satisfy this criterion.

Criterion V: the axon extends no further than can be accounted for by plausible regeneration rates—It was not possible to assess whether L-ALPs satisfied this criterion because none of the L-ALPs could be reconstructed to their terminations. However, it is worth noting that all the reconstructed portions of the L-ALPs were well within the plausible regeneration limits suggested by Steward et al. (2003).

Criterion VI: the axon was tipped with a growth cone—Again, this criterion could not be assessed because the L-ALPs were not reconstructed in their entirety. However, in the 10-day and 8-week survival experiments, many GAP-43-immunoreactive processes were found outside the limits expected for de novo axons arising from dendrites (MacDermid et al., 2002, 2004). These GAP-43-immunoreactive processes may be the growth cones of L-ALPs, but in the absence of more direct evidence, L-ALPs do not satisfy this criterion.

Criterion VII: the axon had an unusual morphology—Two of five L-ALPs had morphologies that are not characteristic of the CINs described by Sugiuchi et al. (1992,

1995) or Matsuyama et al. (2004, 2006). These L-ALPs had ipsilateral collateral branches with irregular varicosities and bouton-like varicosities. In contrast, all collaterals of CINs are restricted to the contralateral ventral horn. However, this evidence in favor of regeneration must be balanced by the seemingly normal morphology of the other three L-ALPs and the fact that there are no comprehensive descriptions of the morphology of all types of CINs in the upper cervical spinal cord.

When our results are assessed from the perspective of all seven criteria described by Steward et al. (2003), we believe there is enough evidence to suggest that L-ALPs may be regenerating axons. However, the match or mismatch to some of the criteria for regenerating axons could not be determined, and some L-ALPs did not satisfy other criteria. Thus, based on the present data, it would be premature to classify L-ALPs definitively as regenerating axons.

Regeneration of CNS axons through a lesioned environment—Much of the previous discussion of regenerated versus spared axons rests on the assumption that axons of CINs, in the absence of extrinsic interventions, are capable of regeneration within a lesioned environment. This assumption contradicts a substantial body of evidence showing that CNS axons are incapable of regeneration through an injury site due to an inhibitory environment (Yiu and He, 2006) and a poor regenerative capacity (Deumens et al., 2005). However, it is also known that motoneurons, axotomized within 0.4–1.4 mm from their soma, can regenerate their axons through a lesion of the spinal cord and reach the ventral roots (Ramón y Cajal, 1959; Risling et al., 1983). This observation is consistent with other studies that have shown that proximal axotomy greatly increases the regenerative capacity of CNS neurons (Richardson et al., 1984; Doster et al., 1991; Fernandes et al., 1999). The injury model used in the present study was specifically designed to axotomize CINs very close to their somata (0.5–3.0 mm). Thus, L-ALPs may be a direct consequence of the high regenerative capacity induced by the proximal axotomy. The finding that some L-ALPs, assuming that they are not spared axons, were found within and beyond the injury site suggests that this enhanced regenerative capacity can overcome the inhibitory milieu of the injury site.

Significance

It has recently been shown that axotomized corticospinal tract axons sprout to form functional connections with intact propriospinal interneurons that bridged the spinal lesion (Bareyre et al., 2004; Vavrek et al., 2006). The results of the present study suggest that interneurons may serve as more than just an alternative route to relay descending signals to spinal neurons caudal to the lesion. Instead, axotomized interneurons in the immediate vicinity of the injury may actively contribute to the construction of new circuits via de novo axons that emerge from distal dendrites. It is not known whether the connections formed by de novo axons that emerge from distal dendrites are functional. If they do form functional synapses, they may represent another means by which interneurons contribute to detours around partial injuries of the spinal cord. The unexpected finding that other axotomized interneurons in the same region had L-ALPs serves as a reminder that proximal axotomy

also triggers an increased regenerative capacity that may be expressed by regeneration of the injured axon through the lesion.

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Fig. 1.

Schematic diagrams of the lesion protocol and Western blot analysis of GAP-43 and MAP2a/b antibodies. **A,B:** Lesion protocol. All lesions began caudally (right) and ended rostrally (left). The leading edge of the blade (a) was hand sharpened, and the trailing edge (b) was the manufacturer's edge. The 5-mm mark was measured from the tip of the blade, along the manufacturer's edge and was perpendicular to the manufacturer's edge. **A:** The trajectories of the first series of penetrations and cuts. Each penetration (i) was 2.5 mm deep, and each rostral movement (ii) was 0.75 mm long. **B:** The trajectories of the second series of penetrations and cuts. Starting at 2.5 mm deep, each penetration (iii) was the distance from 2.5 mm depth until the black mark on the blade disappeared below the surface of the spinal cord, and each cut (iv) was 0.75 mm long. R, rostral; C, caudal. **C,D:** Western blots of GAP-43 antibodies (C) and MAP2a/b antibodies (D). Molecular weight standards (Invitrogen, La Jolla, CA, cat. no. 10748-010) are indicated on the left.

Fig. 2.

Rostral-caudal distribution of Neurobiotin-labeled processes and MAP2a/b-immunoreactive dendrites that crossed the mid-line near five lesions, as seen in non-recovery experiments. **A:** Distribution of Neurobiotin-labeled processes based on one experiment. The distribution of Neurobiotin-labeled processes was reconstructed from serial histological sections that contained the commissures ($n = 10\text{--}13$ sections per experiment). Each section was reconstructed by tracing the section outline, marking the location of the injection sites, and tracing all processes with medial-lateral projections near the midline. These reconstructions were superimposed and aligned by matching the injection sites and the outlines of the reconstructions. The injection sites are indicated by Xs. The Neurobiotin-labeled processes are indicated by the lines immediately below the injection sites. The outer lines indicate the edges of the tissue sections. The dashed gray line indicates the midline. The gray shaded area indicates the rostral-caudal region in which there were no Neurobiotin-labeled processes that crossed the midline. **B–F:** Histograms of the distribution of MAP2a/b-immunoreactive processes and Neurobiotin-labeled processes that crossed the midline in the vicinity of midsagittal lesions. Results from all five experiments are shown. The frequency of MAP2a/b-immunoreactive processes along the midline is expressed as the number of

pixels (black bars) that were immunoreactive for MAP2a/b along a line that followed the midline. All sections that contained the commissures were examined. MAP2a/b immunoreactivity was quantified by using Image Pro Plus. Only those pixels that exceeded the fluorescent intensity of the average fluorescence intensity of several isolated dendrites elsewhere on the section were considered to be MAP2a/b immunoreactive. The gray shaded areas indicate the regions in which no Neurobiotin-stained processes crossed the midline, as determined by the method illustrated in A. In the experiments illustrated in B, E, and F, the rostral-caudal axis of the spinal cord was not parallel to the trajectory of the knife cut. To avoid a superficial cut at one end of the transection and hitting bone at the other end of the transection, the knife cuts were limited to a length of 2 mm. The results shown in B are from the same experiment illustrated in A. R, rostral; C, caudal.

Fig. 3. Distribution of GAP-43-immunoreactive somata. **A:** Epifluorescence photomicrographs of GAP-43-immunoreactive somata. Red indicates GAP-43 immunoreactivity. **B:** Enlarged image of the right soma shown in A. **C,D:** Transverse views of the locations of GAP-43-immunoreactive somata. Dots indicate the location of GAP-43-immunoreactive somata from a 10-day survival experiment in which the cut traversed the left ventral horn (C) and an 8-week survival experiment in which the cut transected the commissures through the midline (D). The path of the lesion, as defined by a series of holes and a disruption in the continuity of the gray and white matter, is indicated by the red line. **E:** Horizontal view of the location of GAP-43-immunoreactive somata 8 weeks following the lesion. R, rostral; C, caudal; dots: locations of GAP-43-immunoreactive somata; gray areas: the location of the gray matter from a representative section; red line: the location of the lesion as defined by the length of the knife cut and placed such that it had the greatest number of GAP-43-immunoreactive somata within its rostral-caudal confines. **F:** Cumulative histograms of the distribution of GAP-43-immunoreactive somata based on data from four experiments: two at 10 days post lesion and two at 8 weeks post lesion. The rostral-caudal distribution of the somata was normalized to the length to the lesion (i.e., length of the knife cut; gray shaded area) and

centered with the greatest number of GAP-43-immunoreactive somata within the gray shaded area. Scale bar = 25 μm in A,B; 1,000 μm in E.

Fig. 4.

Morphological characteristics and distribution of GAP-43-immunoreactive processes. **A–C:** Epifluorescence photomicrographs of simple (A) and complex (B and C) GAP-43-immunoreactive processes. Red indicates GAP-43 immunoreactivity. **D,E:** Distribution of complex (D) and simple (E) GAP-43-immunoreactive processes in the horizontal plane. Based on the same experiment shown in Figure 3D. R, rostral; C, caudal; gray areas: the location of the gray matter from a single representative section; red lines: the location of the lesion as defined by the length of the knife cut and placed such that it had the greatest number of GAP-43-immunoreactive somata within its rostral-caudal confines. **F–I:** Cumulative histograms comparing distributions of GAP-43-immunoreactive somata and simple and complex processes as a function of the distance from the rostral edge of the tissue. **F,G:** Distributions of GAP-43-immunoreactive somata and processes from two 10-day survival experiments. **H,I:** Distributions of GAP-43-immunoreactive somata and processes from two 8-week survival experiments. The gray boxes indicate the length and location of the lesion. The widths of the gray boxes are equivalent to the lengths of the knife cut and are centered with the greatest number of GAP-43-immunoreactive somata within the gray shaded areas. Scale bar = 25 μm in A–C; 1,000 μm in D,E.

Fig. 5.

Partial reconstructions of two de novo axons that emerged from distal dendrites and were GAP-43 immunoreactive. Both reconstructions are shown in the horizontal plane. Distribution of GAP-43 and MAP2a/b immunoreactivity is indicated by the color of the reconstruction (see legend). Dashed lines indicate the origins of other dendritic branches that are not shown. The insets show higher magnification drawings of the de novo axons. **A:** A de novo axon that emerged from a distal dendrite and had an elongated terminal varicosity. This varicosity was GAP-43 immunoreactive. Confocal microscopic images of the regions within the dashed boxes are shown in Figure 6. **B:** A de novo axon that emerged from a distal dendrite and had two bifurcations and three terminal varicosities, two of which were GAP-43 immunoreactive. R, rostral; C, caudal; M, medial; L, lateral. Scale bar = 50 μ m; in A,B; 10 μ m in insets to A,B.

Fig. 6. Distribution of GAP-43 and MAP2a/b immunoreactivity in de novo axons that emerged from distal dendrites. **A,B; D,E:** Confocal microscopic images of GAP-43 immunoreactivity (A), MAP2a/b immunoreactivity (D), and Neurobiotin staining (B,E) of the termination of a de novo axon that emerged from a distal dendrite (upper dashed box in Fig. 5A). **G,H:** Confocal microscopic images of MAP2a/b immunoreactivity (G) and Neurobiotin staining (H) of a proximal dendrite (lower dashed box in Fig. 5A). **C,F,I:** Computer-generated overlays of A and B, D and E, and G and H, respectively. White pixels indicate regions with double labeling. Scale bar = 10 μ m in A–I.

Fig. 7.

A: Partial reconstruction, shown in the horizontal plane, of two de novo axons that emerged from distal dendrites and lacked immunoreactivity for both MAP2a/b and GAP-43. Both de novo axons originated from the same primary dendrite. Distribution of GAP-43 and MAP2a/b immunoreactivity is indicated by the color of the reconstruction (see legend). Dashed lines indicate the origins of other dendritic branches that are not shown. The insets show higher magnification drawings of the entire upper de novo axon (left inset) and the terminal of the lower de novo axon (right inset). **B,C:** Confocal microscopic images of MAP2a/b immunoreactivity (B) and Neurobiotin staining (C) of the region within the dashed box in the right inset of A. **D:** Computer-generated overlay of B and C showing synapse-like contacts between the de novo axon and adjacent dendrites. The white pixels indicate double-labeled regions due to synaptic-like contacts between swellings on the de novo axon and dendrites. R, rostral; C, caudal; M, medial; L, lateral. Scale bar = 50 μm; in A; 10 μm in B (applies to B–D), inset to A.

Fig. 8.

Partial reconstructions of three de novo axons that emerged from distal dendrites and lacked immunoreactivity for both MAP2a/b and GAP-43. Both reconstructions are shown in the horizontal plane. Distribution of GAP-43 and MAP2a/b immunoreactivity is indicated by the color of the reconstruction (see legend). Dashed lines indicate the origins of other dendritic branches that are not shown. The insets show higher magnification drawings of the de novo axons. **A:** An example of a de novo axon that emerged from a distal dendrite and whose morphology resembled the structure of the MAP2a/b-immunoreactive dendrite from which it emerged. **B:** Two de novo axons with multiple right-angled branches and varicosities, which emerged from a dendrite lacking MAP2a/b immunoreactivity. Arrowheads indicate the points at which the proximal processes ceased to have a dendritic morphology. These points were considered to be start of the de novo axons. Arrow indicates the path of the de novo axon that starts at the lower arrowhead. R, rostral; C, caudal; M, medial; L, lateral. Scale bar = 50 μ m; in A,B; 10 μ m in insets to A,B.

Fig. 9.

A: Partial reconstruction, shown in the horizontal plane, of an L-ALP that projected to both sides of the spinal cord. For clarity, the dendrites are not shown. Gray area represents the location of the gray matter as seen at the dorsal-ventral level of the soma. The rostrally and caudally projecting branches of the L-ALP in the white matter extended beyond the region shown in this reconstruction. Distribution of GAP-43 and MAP2a/b immunoreactivity is indicated by the color of the reconstruction (see legend). The insets show higher magnification drawings of fine-diameter branches that emerged from the L-ALP. **B,C:** Confocal microscopic images of MAP2a/b immunoreactivity (B) and Neurobiotin staining (C) of the region within the dashed box. **D:** Computer-generated overlay of B and C. The white pixels indicate double-labeled regions due to synaptic-like contacts between swellings on the L-ALP and dendrites. R, rostral; C, caudal. Scale bar = 250 μ m; in A; 20 μ m; in left inset to A; 10 μ m in B (applies to B–D) and right inset to A.

Fig. 10.

Schematic summary of reconstructed L-ALPs. **A–C:** L-ALPs reconstructed from experiment shown in Figure 11A,C. **D,E:** L-ALPs reconstructed from experiments shown in Figure 11B,D. Black circles represent somata. Gray shaded areas represent the ventral gray matter. Dashed line represents the midline. R, rostral; C, caudal.

Fig. 11.

Rostral-caudal location of L-ALPs that crossed the mid-line relative to the distribution of dendrites that crossed the midline and the distribution of GAP-43-immunoreactive somata. Data from two experiments are shown. **A,B:** The distribution of MAP2a/b-immunoreactive processes that crossed the midline. The methods used to construct these histograms are identical to those used in Figure 2. Three of the four reconstructed L-ALPs that crossed the midline were found in the experiment shown in A. These L-ALPs crossed the midline in a narrow rostral-caudal band, 100 μm long. The position of this band is indicated by the arrow. The remaining reconstructed L-ALP that crossed the midline was found in the experiment shown in B. The arrow indicates the location at which this L-ALP crossed the midline. **C,D:** Cumulative histograms of the rostral-caudal distribution of GAP-43-immunoreactive somata from the experiments shown in A and B, respectively. The gray boxes indicate the length and location of the lesion. The widths of the gray boxes are equivalent to the lengths of the knife cut and are centered with the greatest number of GAP-43-immunoreactive somata within the gray shaded areas. The arrows indicate the locations at which the L-ALPs crossed the midline.

TABLE 1

Frequency of GAP-43-Immunoreactive Somata and Processes[/]

Experiment	Survival time	Length of knife cut (mm)	No. of GAP-43 somata	No. of complex GAP-43 processes	No. of simple GAP-43 processes	% of simple GAP-43 processes within the rostral-caudal region of the lesion site
CI-4	10 days	3.75	33	103	169	26
CI-5	10 days	3.00	65	32	235	20
CI-7	8 weeks	3.50	639	181	547	54
CI-8	8 weeks	3.50	446	58	310	51

[/]No precautions were taken to avoid double counting of processes and somata that may have been split by histological sectioning. GAP-43, growth-associated protein-43.

TABLE 2

Summary of Reconstructions¹

	CINs with de novo axon(s) that emerged from distal dendrites	CINs with L-ALPs	CINs with no de novo axons that emerged from distal dendrites or L-ALPs	Total
No. of CINs	4	5	1	10
Dendritic arbores reconstructed	16/17 ²	22/25 ²	2/2 ²	40/44 ²
Dendritic arbores with at least one de novo axon that emerged from a distal dendrite	8	0	0	8
Dendritic arbores without MAP2a/b immunoreactivity	2	0	0	2
De novo axons that emerged from distal dendrites	25 ³	0	0	25 ³
L-ALPs	0	5	0	5

¹Dendritic arbores refer to a primary dendrite and all of the dendritic segments that emerge from that primary dendrite. CINs, commissural interneurons; L-ALPs, long axon-like processes; MAP2a/b, microtubule-associated protein2a/b, GAP-43, growth-associated protein-43.

²Reconstructions following GAP-43 processing/reconstructions following MAP2a/b processing.

³The distribution of GAP-43 immunoreactivity was not recorded for three of these *de novo* axons that emerged from distal dendrites.