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*Article*

# Observations on Lumbar Spinal Cord Recovery after Lesion in Lizards Indicates Regeneration of a Cellular and Fibrous Bridge Reconnecting the Injured Cord

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**Abstract**: The lumbar spinal cords of lizards were transected, but after the initial paralysis most lizards recovered un-coordinated movements of hind limbs. At 25-45 days post-lesion about 50% of lizards were capable of walking with a limited coordination. Histological analysis showed that the spinal cord was transected and the ependyma of the central canal formed two enlargements to seal the proximal and distal ends of the severed spinal cord. Glial and few small neurons were formed while bridge axons crossed the gap between the proximal and the distal stumps of the transected spinal cord as was confirmed by retrograde tract-tracing technique. The bridging fibers likely derived from interneurons located in the central and dorsal grey matter of the proximal spinal cord stump suggesting they belong to the local central locomotory pattern generator circuit. The limited recovery of hind limb movements may derive from the regeneration or sprouting of short proprio-spinal axons joining the two stumps of the transected spinal cord. The present observations indicate that the study on spinal cord regeneration in lizards can give insights on the permissive conditions that favor nerve regeneration in amniotes.

**Keywords:** lizard; lumbar spinal cord; transection; regeneration; histology; tract-tracing

## 1. Introduction

In most vertebrates, lesion of the spinal cord (SC) determines various degrees of paralysis, with notable exceptions in cyclostomes, some teleost fish, and aquatic salamanders [1–6], large injuries in the SC of mammals and birds determine permanent paralysis [7,8], and this result was also reported for reptiles [9,10]. Past studies on extant reptiles, such as lizards [11,12], and recent studies on turtles [13,14] have, however, shown that the lumbar and thoracic segments of the SC in reptiles can partially recover functionality and some anatomical connections.

The regeneration of the SC in lizard occurs in the tail as part of the general process of tail regeneration [9,15–17]. The regenerated tail SC is very simplified and centered around the proliferating ependymal tube, which is surrounded by some hundreds of axons mainly originated in the original SC in continuation with the regenerated cord [10]. The ability to regenerate the caudal SC seems related to the presence of a growing tail while no regeneration in more rostral parts of the SC has been reported for the iguanid lizard *Anolis carolinensis* [9,10].

Previous observations on the lacertid lizard *Podarcis sicula*, however, indicated that, after the initial paralysis during the first two weeks post-transection of the dorsal (thoracic) spinal cord, partially coordinated movements of the hind limbs were restored [11,18,19]. In some cases, the histological analysis of the SC in lizards capable of recovering some movements of the hind limbs indicated that the SC lesion was incomplete [18]. In these cases probably the intact nervous fibers were able to maintain some anatomical and physiological connectivity between the proximal and distal stumps adjacent to the interrupted SC. However, cases of completely severed SC also managed to recover some movement of the hind limbs [11,19]. These lizards use their hind limbs voluntarily to escape capture and this observation indicates that the movement derives from the formation of a bridge tissue that re-connects the lesioned spinal cord. The reconnecting axons possibly re-activated the intrinsic circuits of the "central locomotor generator" present in the spinal cord [20–23], whihc was initially interrupted from the transection. This process of recovery was previously described in the spinal cord of fish [2] and salamanders [3,4].

In lizards, as representative of low amniote vertebrates, no proof is presently available about the possibility to restore connections with the intrinsic patter locomotor generator system of the lumbar SC after lesion. In order to address further the knowledge on the possibility of regeneration in more rostral regions of the lizard SC, the present qualitative histological and tract-tracing study has extended the analysis of the process of SC recovery in lizard after injury of the lumbar SC. As a part of a broader study on the influence of nerve regeneration to the process of tail regeneration in lizards, the present study was aimed to assess a possible influence of the degeneration of descending spinal nerves on tail regeneration. However, the final goal of the present study is to determine the histological recovery of the injured SC, the presence or absence of scar tissue in the point of lesion, and determine whether some axons can actually cross the lesion point and restore the connectivity of the transected lumbar SC, a process that could explain the recovery of some movements in the hind limbs.

## 2. Materials and Methods

### *2.1. Experimental Procedure*

Adult lizards (*Podarcis muralis*) of both sexes but unknown age were utilized in the study, with sizes ranging from 16 cm to 20 cm. The animals were collected in early Summer in the outskirts of Padua, Italy, and maintained at seasonal conditions and daily natural light (25–30 °C during daytime). All the surgical and post-operative procedures followed the regulations on animal care and experimentation procedures under the Italian and European Guidelines (art. 5, DL 116/92).

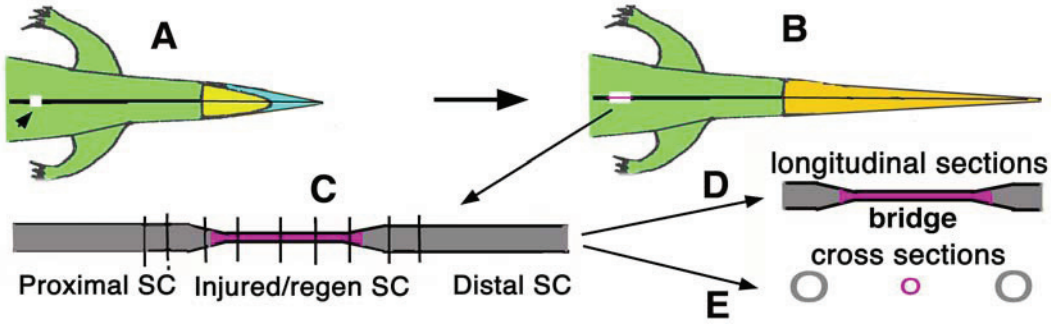
A first experimental group consisted of 25 lizards that were utilized for the analysis of the behavioral recovery followed by the histological analysis of the injury and variably recovered SC. The animals were anesthetized with ethylic ether after they were confined for 1–3 minutes in a chamber with saturated ether vapors before surgery. Both the scissors, scalp and fine tweezers used for cutting, as well as the lumbar part of the skin of the back were disinfected with 70° ethanol: Lugol joidine solution (1:1 v/v). The integument of the back in the lumbar region was cut open 0.5–1.0 cm using a sharp scalpel. The skin was kept on the sides in order to have a sufficient subcutaneous area to operate. Dorsal and inter-vertebral muscles were sectioned until the vertebral column was visible. The vertebral column was held in place using firm tweezers for the following incision. Bleeding was generally minimal at this stage and blotted using clean blotting paper. Using a sharp scissor a quick snap-cut was made between the 3rd and the 4th lumbar vertebra from the pelvic girdle, as schematically shown in Figure 1A. The transection of the spinal cord was initially revealed by the free whip-like undulation of the tail and the transection was also checked visually by inspection under a stereomicroscope before some bleeding obscured the transected area. The dislocated vertebrae were re-aligned and the severed muscles were recomposed over the vertebrae. The bleeding that generally occurred after this operation was in part absorbed using clean blotting paper. The lesion tissues and the skin tissues were covered with a wound cicatrizing powder containing antibiotics (Cicatrene, Welcome Italia, Pomezia-Rome), and the skin was recomposed over the underlying tissues. No sutures were made and the borders of the skin remained trapped within the clot that was covered by the cicatrizing powder. The bleeding rapidly stopped and more cicatrizing powder was administered over the following two-three days to keep the tissues of the wounded area not directly exposed to the environment while forming the scab. After this operation, the animals also had their tail transected for allowing tail regeneration (Figure 1A,B) during the entire span of the experiment (29, 36 or 45 days). After sacrifice of the lizards, the SC with the surrounding vertebrae was collected, fixed and sectioned in both transversal and longitudinal section as schematically shown in Figure 1C,D.

A second experimental group consisted in six lizards with regenerating tails (about 4 mm long) underwent the transection in the lumbar SC as indicated above, and these samples were utilized for a tract tracing study using the Dil tracer. The animals were sacrificed at the 36th day post-lesion, and the operated area was removed and fixed in 4% paraformaldehyde for 2 days at 4 °C. The fixed tissues consisted in 6–7 mm by 3 mm pieces of the vertebral column containing 2 non-injured vertebrae proximal and 2 non-injured vertebrae distal to the level of lesion. After fixation the SC was freed from the surrounding bones of the vertebral column showing continuity (bridge) between the two stumps (rostral and caudal) as schematically illustrated in Figure 1C. The recovered SC was maintained wet in

the same fixative as above. Using fine tweezers, some crystals of the lipophylic fluorescent tracer Dil (molecular Probes, Eugene, OR, USA) were inserted into the distal SC trunk, located at 2–3 mm from the transected SC. The entire preparation was maintained immersed in the fixative into eppendorf vials and stored in the dark at 4 °C for 40 days.

The preparation was then removed from the fixative, and the SC was embedded in 5% agar and sectioned with a vibratome (Lancer, Series 1000) in cross-sections of 50–60 μm in thickness. The sections were collected serially over slides and mounted in 3% glycerine in 0.1 M Phosphate Buffer at pH 7.2. The sections were observed under a Fluorescent microscope using a Fluoresceine filter, and photographed with a digital camera (Euromex-500, The Netherlands).

**Figure 1.** Schematic drawing showing the experiment presented in the study. The lumbar spinal cord was transected (arrowhead in **A**) in lizard with regenerating tail (**B**). **C** shows the two stumps of the transected cord (grey color) and the thinner gap indicating the regenerated/repaired spinal cord (violet color). Both longitudinal (**D**) and progressive cross-sections (**E**) of the stumps spinal cord and of the repaired/regenerated gap were analyzed (see methods).




*2.2. Fixation and Microscopic Methods*

The entire lesion region of the vertebral column from lizards of the first group, including the transected spinal cord and vertebrae, with at least 2 non-injured proximal and distal vertebrae, was fixed in 3% glutaraldehyde in Phosphate buffer 0.1 M at pH 7.4 for about 48 h at 4 °C. The long fixation period was chosen in order to fix en-block the large pieces of the injured area, pieces that measured about 6–7 mm in length and were 2–3 mm wide. The vertebrae were in part cut to allow the rapid penetration of the fixative. The tissues were rinsed in buffer for about 2 h, and post-fixed for 3 h in 2% Osmium tetroxide in water, dehydrated in ethanol and infiltrated in propylene oxide for about 3 hours, and then in a 1:1 mixture of propylene oxide and Araldite resin for 1 day at room temperature. The tissues were then embedded for 48 h in pure Araldite resin before curing the blocks for 3 days at 60 °C. This procedure aimed to infiltrate all the very heterogeneous tissues of the sampled area that also included the bone tissue of the vertebrae.

The embedded tissues were sectioned with an ultramicrotome at 1–3 μm in thickness in either longitudinal plane (1 case from 29 days and 2 cases from 36 days post-lesion) and in cross-sections (5 cases from 29 days, 4 cases from 36 days, and 3 cases from 45 days post-lesions). For the longitudinal sectioning, about 10 plastic sections were collected on slides every 50–60 μm starting from the external part of the SC and progressing toward the central, medial part of the SC. For the serial

collection of sections in the cross plane the proximal (rostral) stump of the SC was sectioned proceeding toward the transected area and then continuing into the distal (caudal) stump of the SC. During the sectioning, about 10 plastic sections were collected every 60–70 μm progressing through the entire SC, and in the recovered area between the proximal and distal SC stumps, about 5 sections were collected every 30–40 μm. The sections were stained using 1% Toludine blue, dried and permanently mounted in Eukitt mounting medium (Kindler, GmbH & CO, Freiburg, Germany).

## 3. Results

### *3.1. Qualitative Observations on the Post-Amputation Behavioral*

In the first group, after SC transection, the hind limbs of the 25 animals remained rigid and extended backward for one to two days. After this period the limb became flaccid (forceless) but remained extended backward, and could not move up to 11–17 days. Eight lizards died between seven and 20 days post-amputation, probably in relation to the surgical intervention, but 17 animals survived until sacrifice for the histological analysis of the spinal cord. After more than 17 days post-operation the lizards were capable of righting themselves when positioning lying on their back. The latter ability, to turn the body from an inverted position to the normal position, was very difficult or unfeasible at 2–10 days post-lesion. Besides, at 29, 36 and 45 days post-injury, the stepping movements noted in lizards could be induced by stimulating an escaping reaction after tapping on the cage wall or trying to grab the lizards. Apparently, there was no difference in recovery between males and females.

At 29 days post-amputation six animals were sacrificed for the microscopic study: all the animals were able to move their hind limbs, making flexion and extensions when touched. However two of these lizards also used the hind limbs to help actively the stepping movement (good recovery) while the remaining four in part dragged at least one of their hind limbs (modest recovery). Another six animals were sacrificed at 36 days post-amputation for the histological examination of the injured SC. Also in these cases, all the animals showed irregular movements of their legs but in four cases the body movement (stepping) was not linear and some drag of at least one limb was noted (modest recovery) while two individuals moved the legs rapidly helping the forward movement of the body along a horizontal plane (good recovery). At 45 days post-injury, the last five surviving animals were also sacrificed for the histological study. Three animals showed a good recovery but other two cases still dragged at least one limb during the stepping movements (modest recovery). Despite the irregular movements all these lizards at 29, 36 and 45 days post-lesion moved faster in the cage then during the first 17 days post-operation. The lizards from 29, 36 and 45 days post-operation were, however, unable to lift up on their legs when they stood on the vertical wall of the cage, indicating poor muscle strength in their hind limbs after these periods.

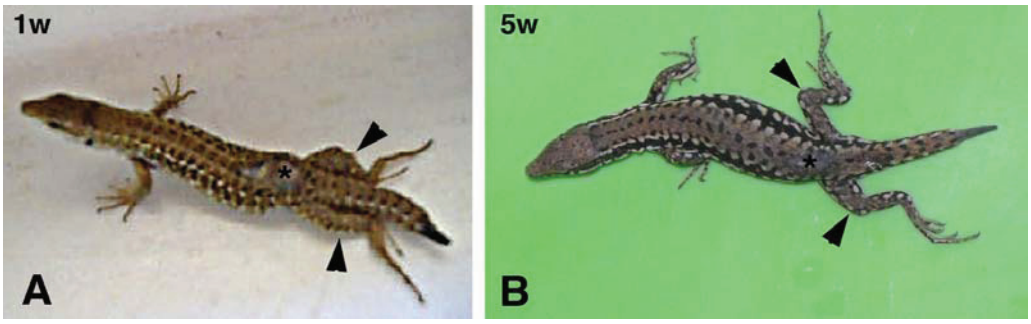
In the second group (six lizards) after seven days, the rigid paralysis was evident and the animal drag the stiff hind limbs that remained extended in a posterior direction (Figure 2A). After 15 days from the lesion no lizard had recovered hind limb movements and two lizards had died, probably for unknown post-operative complications. The four surviving lizards at 36 days post-operation showed a variable degree of hind limb recovery (Figure 2B). One female and two males showed some recovery of hind limbs folding (flexion and extension) but their movement was irregular and often they dragged

at least one limb (modest recovery). The remaining male could make a flexion movement in the two hind limbs, and this helped the animal to move fast along a horizontal plane (good recovery). No convincing vertical movement, capable of lifting these lizards up on the vertical wall of the cage using the hind limbs, was observed in these lizards.

### *3.2. Cases Utilized for the Histological Observations (1st Group)*

The examination of longitudinal sections of the bridge spinal cord at 29, 36 and 45 days post-transection showed similar aspects (Figure 3). In a case that showed some functional (modest) recovery after 29 days, the neural arc and the vertebral body in the injured vertebrae showed broad areas occupied by cartilage or by a cellular (immature) bone, all signs that indicated a high level of connective, cartilaginous and even bone tissue restoration in lumbar vertebrae (Figure 3A).

**Figure 2.** Examples of two lizards after one week (**A**) and five weeks (**B**) post-transection (asterisks indicate the point of transection). In **A** note the stiff hind limbs directed caudally (arrowheads). Note in **B** the folded hind limbs (arrowheads) showing a walking movement.




The bridge tissues located between the larger proximal and distal SC stumps contained numerous cells and fibrous bundles that joined the two stumps (Figure 3A,B). Some of the bundles resembled nerve fascicles containing glial cells, and these bundles were stained dark after the osmium solution utilized to fix the tissue. The fibrous bundles stood out a paler and irregular dense connective tissue, made of oriented or more irregular fibrocytes, and resembling a scar connective tissue (Figure 3B,C). This connective tissue appeared to occupy most of the space in the neural canal located peripherally to the bridge tissue in which the ependymal canal formed two expanded terminal ampullae, one belonging to the proximal and the other to the distal stump of the spinal cord (Figure 3C). The two ependymal ampullae were surrounded or in continuity with some of the fibrous nerve bundles of the stumps.

In another case at 36 days post-lesion with some hind limb movements (modest recovery), terminal ependymal ampullae were also seen in the distal (Figure 4A,B) and in the proximal (Figure 4C–E) spinal cord stumps. However, the injured vertebrae were largely dislocated while large part of the vertebral canal appeared obstructed from the proliferation of cartilaginous tissue (Figure 4C). The partial obstruction of the spinal cord canal was therefore reduced from the broad cartilage regeneration that had occurred to repair part of the injured vertebral bones following SC transection. Few and small threads of nervous aspect were, however, seen crossing the bridge tissue. Despite of this mechanical trap, many neurons in both distal and proximal stumps were present, although their cytoplasm showed clumped Nissl substance, indicative of cell damage (Figure 4B,E). Numerous vacuolated spaces in the white matter indicated a massive axonal degeneration in both stumps of the transected SC.

**Figure 3.** Histological longitudinal section showing the restored spinal cord at 29 days post-lesion. **A**, general view of the connective tissue and of the nervous bridge located between the two stumps of the spinal cord. Bony fragments of the neural arch (arrowheads) that was cut by the surgical intervention to reach the spinal cord. Arrows point to nervous bundles crossing the bridge. Scale bar, 0.2 mm. **B**, detail on the glial-connective tissues present in the gap region with sparse bundles of nervous fibers (arrows). Scale bar, 0.1 mm. **C**, other detail of the lesion spinal cords showing the proximal and distal ependymal dilatations (ampullae) separated by the glia-connective tissues filling the gap (arrows indicate fibrous/nerve fibers; double arrows indicate fibers in continuity with the two ependymal ampullae). Scale bar, 0.1 mm. Legends: bm, bone marrow; de, distal ependymal ampulla; ds, distal spinal cord stump (downstream of the lesion); iv, inter vertebral cartilage; pe, proximal ependymal ampulla (enlarged); ps, proximal spinal cord stump (rostral to the lesion); rc, regenerated cartilage (vertebral repair); rs, regenerated/repaired spinal cord; v, vertebral body.

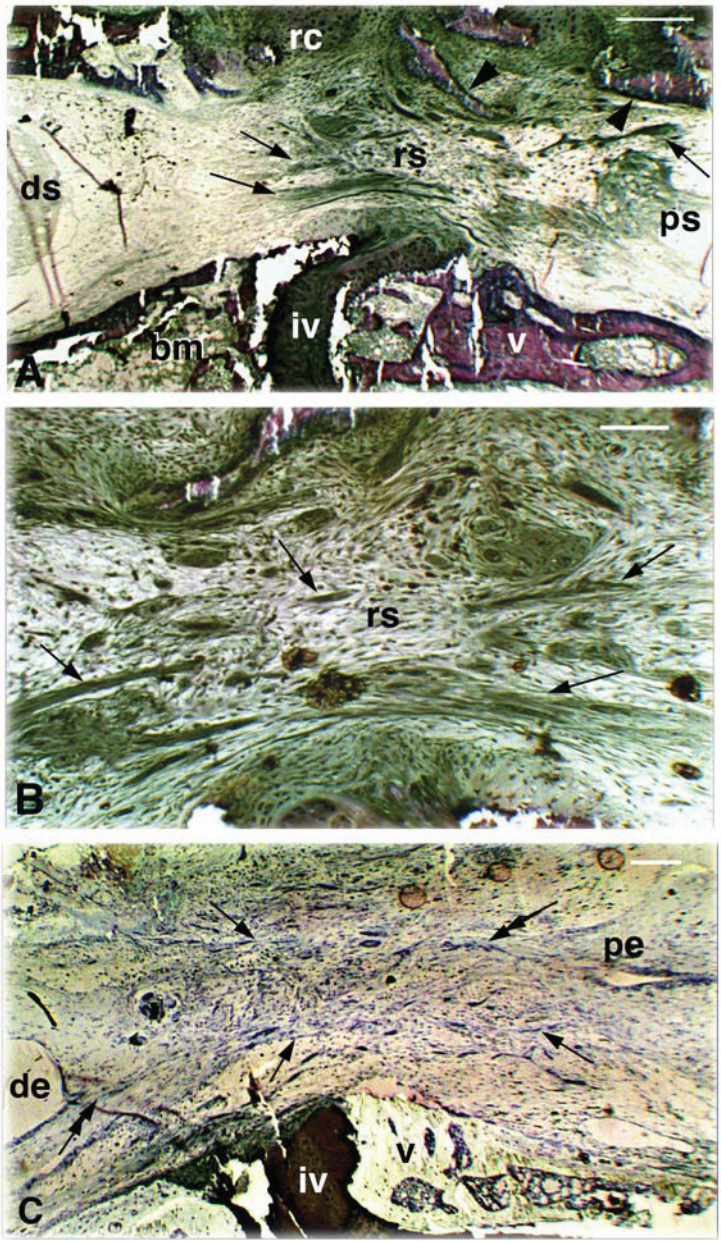
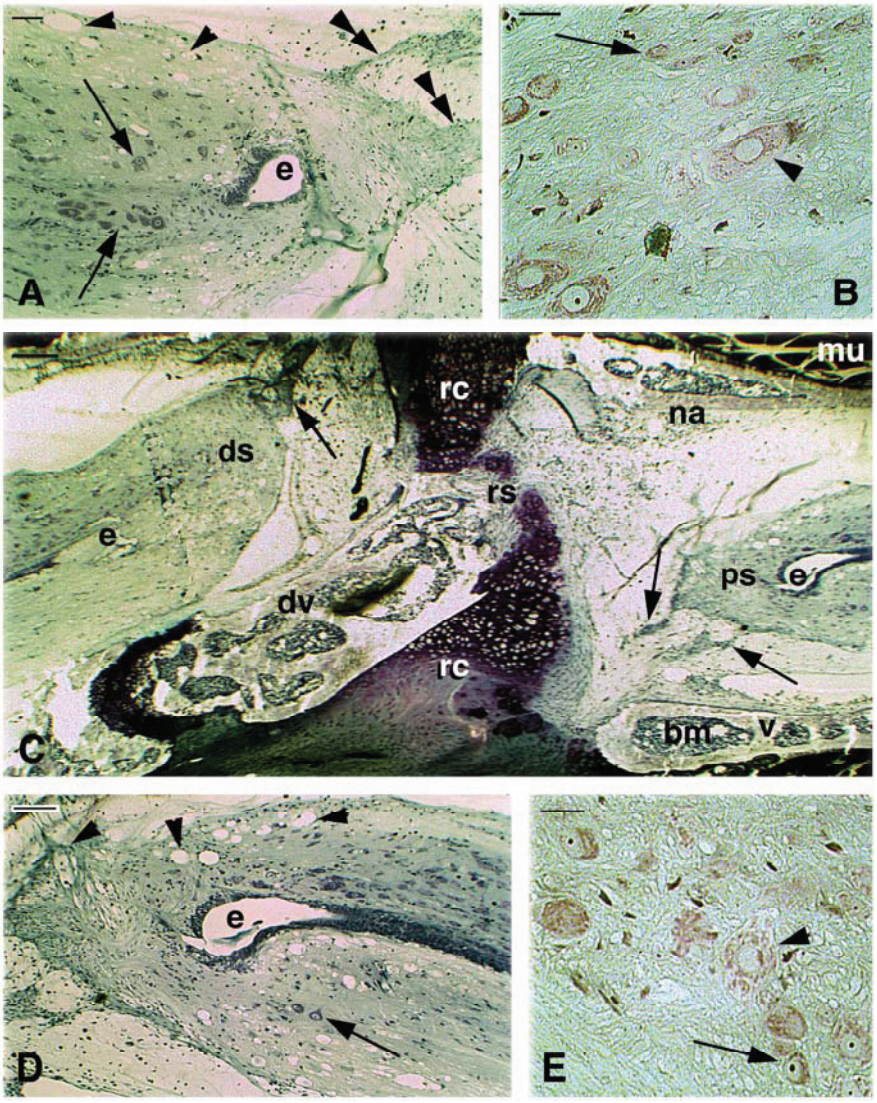


**Figure 4.** Detail of longitudinal sections from an animal with little recovery at 36 days post-lesion. **A**, distal stump showing the enlargement of the ependymal canal and numerous apparently normal neurons (arrows). Arrowheads indicate vacuolated areas resulting from neuropile/axonal degeneration. Double arrowheads point to the beginning of the bridge tissue. Scale bar, 30 μm. **B**, cytological detail of interneurons (arrow) and larger nerve cell bodies (arrowhead) containing granulated Nissl material, located in the distal stump of the spinal cord. Scale bar, 10 μm. **C**, gap region between proximal and distal spinal cord stumps showing that a dislocated vertebra (through the surgical procedure) has largely obliterated the neural canal together the regenerated cartilage. The arrow indicates the neural arch bone. The arrowhead indicates the bone of the vertebral body. Scale bar, 0.1 mm. **D**, enlargement of the ependymal ampulla formed in the proximal spinal cord stump. Note the numerous vacuolated degenerating axons (arrowheads). Scale bar, 20 μm. **E**, detail of the proximal stump showing large ventral neurons (arrowhead) and sparse smaller neurons (arrow) containing coarse Nissl bodies, a typical sign of neuron reactivity to axotomy. Scale bar, 10 μm. Legends: bm, bone marrow; dv, displaced vertebra; ds, distal stump of the spinal cord; e, ependyma; mu, muscles; na, neural arch; ps, proximal stump of the spinal cord; rc, regenerated cartilage.



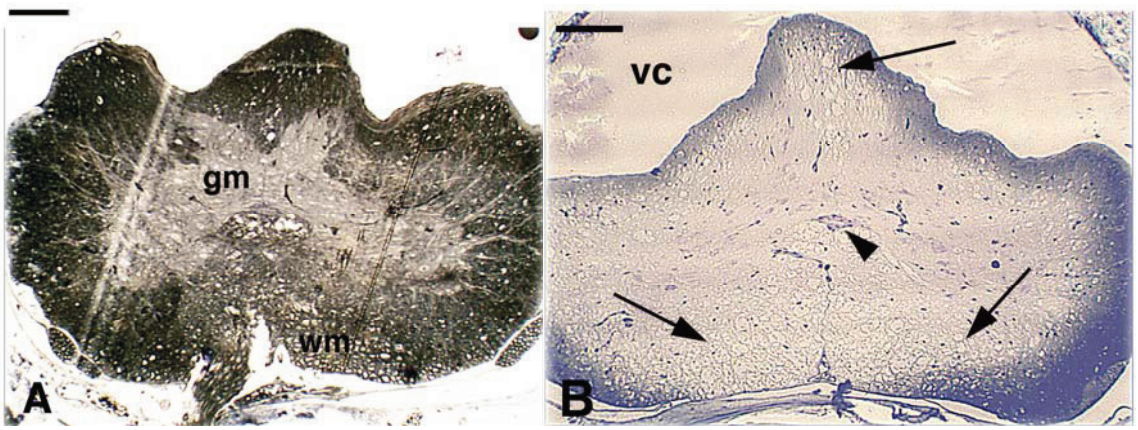
Similar results were observed from the study of sequential cross-sections from the proximal spinal cord stump and across the bridge to reach the distal spinal cord stump. The observations of cross sections produced better information on the degree of white matter degeneration, in terms of distribution of degenerated (vacuolated) fibers in the entire section of the SC. In a case with good recovery at 29 days post-injury, the proximal stump located at about 1–2 mm from the lesion, showed that most axons appeared still myelinated (Figure 5A). Approaching the bridge tissue, below 1 mm from the lesion area, broad areas of degenerating axons became evident, especially in the ventral and dorsal tracts of the white matter (Figure 5B,C). Also the central (ependymal) canal became enlarged and the nuclei of ependymal cells were more evident. Although many neurons were still present in the proximal SC stump, the outline of the grey matter became more and more indistinct and irregular in more distal sections approaching the bridge region (Figure 5C) while the reduced white matter contained numerous small and large degenerating nervous fibers or areas containing degenerated axons (Figure 5D). The central canal appeared four- to six-folds larger then in the proximal sections more distant from the bridge, with a lumen larger than 40 μm and a partly stratified and hypertrophic ependyma (Figure 5E). Among elongated ependymal cells, few roundish and basophilic cells (arrowhead in Figure 5E) or pale cells with a large nucleolus (arrow in Figure 5E) were seen. Numerous small (glial) cells were seen in the white matter, among the degenerated axons.

In the more central, thinner or irregular part of the bridge spinal cord (about 200 μm thick), neither neurons nor ependyma were seen but instead numerous pale areas, representing axonal and neuropilar tissues, were present among glial cells and degenerating axonal spaces (Figure 5F,G). This bridge tissue consisted of irregular glial-fibrous connective, resembling scarring connective for the abundance of glial or fibrocyte cells. Progressing distally, beyond the level of transection, the distal spinal cord stump regained, eventually, a similar diameter as in the proximal spinal cord, but few and smaller motorneurons were seen in the ventral grey horns, suggesting that most of these cells had degenerated after transection (Figure 5H).

In the five cases sacrificed 45 days post-operation, histological features similar to those described at 29 and 36 days post-transection were observed. In an animal with a good recovery, numerous myelinated axons disappeared while they were replaced by vacuolated spaces (Figure 6A,B). Besides, the presence of stained clumps in the cytoplasm (chromatolysis) in many neurons of the grey matter indicated cell damage. The enlargement of the central canal was also noted in the proximal spinal cord approaching the bridge, while the grey matter became less clearly defined and vacuolated axons appeared quite numerous in the ventral, lateral and dorsal columns of the white matter (Figure 6C). In cross sections of the proximal bridge area, the anatomical structure of the spinal cord was lost, the ependymal canal was much enlarged while few neurons were seen in the irregular grey matter and numerous small (glial) cells were present in the white matter (Figure 6D). The bridge was often surrounded by a scarring connective that occupied a large part of the vertebral canal and that internally contacted the nervous tissue and externally the dura meninges or the periosteum of the vertebrae (Figure 6E). Progressing distally toward the medial region of the bridge, although the central canal appeared narrow (Figure 6E), the ependymal wall was still stratified, featuring denser (basophilic) or paler cells among the elongated ependymal cells (Figure 6F).

In more distal regions, past the transection level, the collected serial sections progressively intercepted the distal stump of the spinal cord where the nervous tissue regained the normal size and showed the typical organization in a distinct white and grey matter, although the distal stump remained thinner when compared to the proximal stump of the transected spinal cord. The ependymal canal became initially larger again (corresponding to an ependymal dilatation or ampulla) and sparse vacuolated areas were apparent in the surrounding white matter (Figure 6G). In the distal stump of the spinal cord, however, rare large motorneurons were seen in the ventral grey horn or they were absent. The central canal of the distal SC past the bridge appeared reduced like in the normal, proximal stump of the spinal cord (Figure 6H).

**Figure 5.** Representative proximal-distal cross-sections of lesion spinal cord at 29 days post-injury. **A**, proximal spinal cord showing the characteristics H-shapes of the grey matter surrounded by numerous myelinated axons in the white matter. Scale bar, 0.1 mm. **B**, a more caudal section closer to the transected region but still in the proximal SC stump shows numerous vacuolated axons (arrows) in the white matter. The arrowhead indicates the ependyma. Scale bar, 0.1 mm. **C**, a further caudal section shows the presence of large vacuolated areas (arrows) previously occupied by axons. The arrowhead indicates the ependyma. Scale bar, 0.1 mm. **D**, section of the reduced SC in the bridge region with the enlarged ependymal canal (arrowhead) while the distinction between grey (very reduced) and white matter appears irregular. Numerous degenerated axons are seen (arrows) and the SC is occupied by numerous glial cells. Scale bar, 0.1 mm. **E**, detail of the stratified ependymal canal with indicated pale cells (arrows) among ependymal cells. Another cells with basophilic cytoplasm is seen nearby the ependyma (arrowhead). Scale bar, 15 μm. **F**, section across the bridge region between the two SC stumps formed by a scarring glial tissue (arrow), sparse nerve bundles and degenerating axons (arrowheads). Scale bar, 0.1 mm. **G**, more caudal part of the bridge showing the presence of bundles of nerves (arrows) within the surrounding dense connective (scar). Scale bar, 0.1 mm. **H**, distal spinal cord with the typical H-shaped grey matter surrounded by numerous degenerated axons in the white matter (arrows). The ependymal camnal (arrowhead) appear reduced as in the proximal stump. Scale bar, 0.1 mm. Legends: bv, blood vessel; e, ependyma; gm, grey matter; mx, meninx; n, neuron; na, neural arch; rc, regenerated cartilage; sc, spinal cord; sco, scarring connective-meninx; sn, small neuron; v, vertebra; vc, vertebral canal; wm, white matter.



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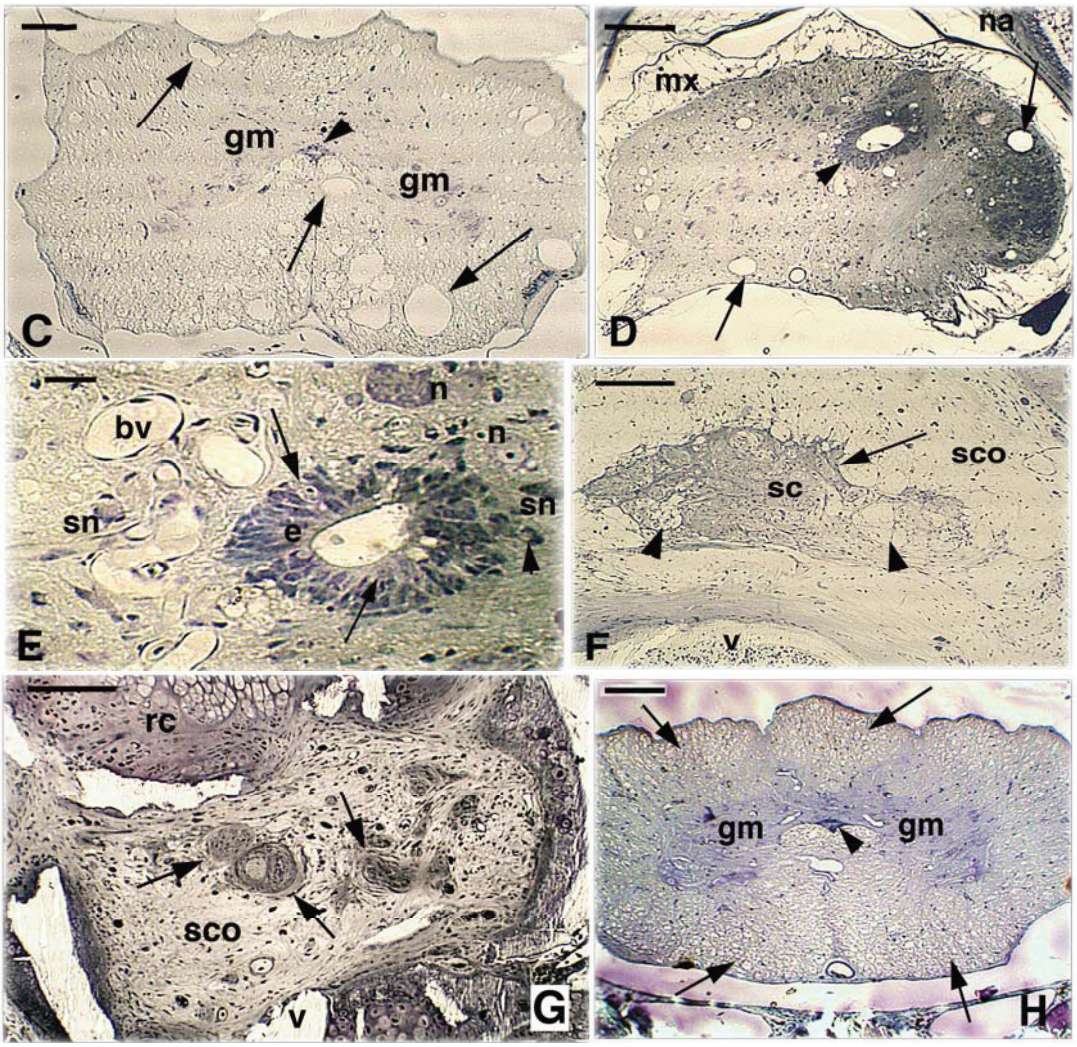



*3.3. Cases Utilized for the Dil Tract-Tracing Study (2nd Group)*

After 15 days from the lesion, no lizard had recovered hind limb movements and two lizards died; possibly as the result of the surgical intervention. The four survival lizards at twenty-six days showed a variable degree of recovery of hind limb movement. One female (case #1) and two males (cases #3 and #4) showed some recovery (modest recovery) of hind limb folding (flexion and extension), but one male (case #2) could make a flexion movement in both hind limbs that helped the stepping movements (good recovery). The animals were all sacrificed at the 36th days post-lesion, and the operated area was removed and fixed as previously indicated for the application of the Dil fluorescent tracer.

The observations of the extracted spinal cord in the four cases, before the Dil application, showed that case #1, with a modest recovery of hind limb motility, was discontinuous in the dorsal part of the SC so that about half of the SC was actually continuous between the two stumps. This bridge probably represented regenerated tissue, since the transection was completed. Case #2, also featuring a limited recovery, showed the presence of a thin tissue bridge formed at 36 days post-transection, likely regenerated, present between the two stumps. The other two cases with a relative recovery of the limb mobility (modest and good recovery) showed that the SC was still largely discontinuous between the two stumps, aside the more ventral part of the SC where a thin bridge tissue, likely regenerated, was present.

**Figure 6.** Representative proximal distal cross sections of spinal cord 36 days post-lesion. **A**, proximal spinal cord has the normal H-shape of the grey matter and numerous axons in the white matter are myelinated. Scale bar, 0.1 mm. **B**, a more caudal section closer to the bridge region showing a reduction in diameter and the presence of numerous degenerated axons (arrows). The arrowheads point to neurons within the grey matter. Scale bar, 20 μm. **C**, a more caudal section of the proximal stump spinal cord shows a further reduction of the white matter, flattening and vacuolization (arrows) while numerous cells are present in the grey matter (arrowheads). Scale bar, 0.1 mm. **D**, more caudal section at the level of the ependymal ampulla that appears surrounded by few neurons and nerve fibers of the white matter. Some vacuoles (arrow) indicate degenerated areas. Scale bar, 0.1 mm. **E**, further caudal section in the bridge region showing a narrow spinal cord with a reduced ependymal canal (arrowhead) while the irregular white matter contains numerous glial cells and fewer neurons. Abundant scar tissue is seen between the spinal cord and the vertebral bone, probably derived from the surgical incision made to reach the spinal cord to perform the transection. Scale bar, 0.1 mm. **F**, close-up on the stratified ependymal epithelium present in the bridge region. Around the reduced central canal numerous stratified cells from the ependyma are seen, including dark round cells (arrowheads), pale cells (double arrowheads) representing neural or glial cells. Scale bar, 25 μm. **G**, caudal section of the spinal cord stump after the bridge, showing the ependymal canal still enlarged (arrowhead) surrounded by a larger number of neurons and white matter not yet distinct into grey and white matter. Arrows indicate the larger degenerated axons. Scale bar, 0.1 mm. **H**, caudal most level of the distal SC stump that shows increased diameter. Arrowheads indicate the grey matter with numerous neurons, but small or large (arrow) vacuolated areas are present. Scale bar, 0.1 mm. Legends: c, central canal; e, ependyma; gm, grey matter; mx, meninx; sc, spinal cord; sco, scarring connective-meninge; v, vertebral bone; vc, vertebral canal; wm, white matter.

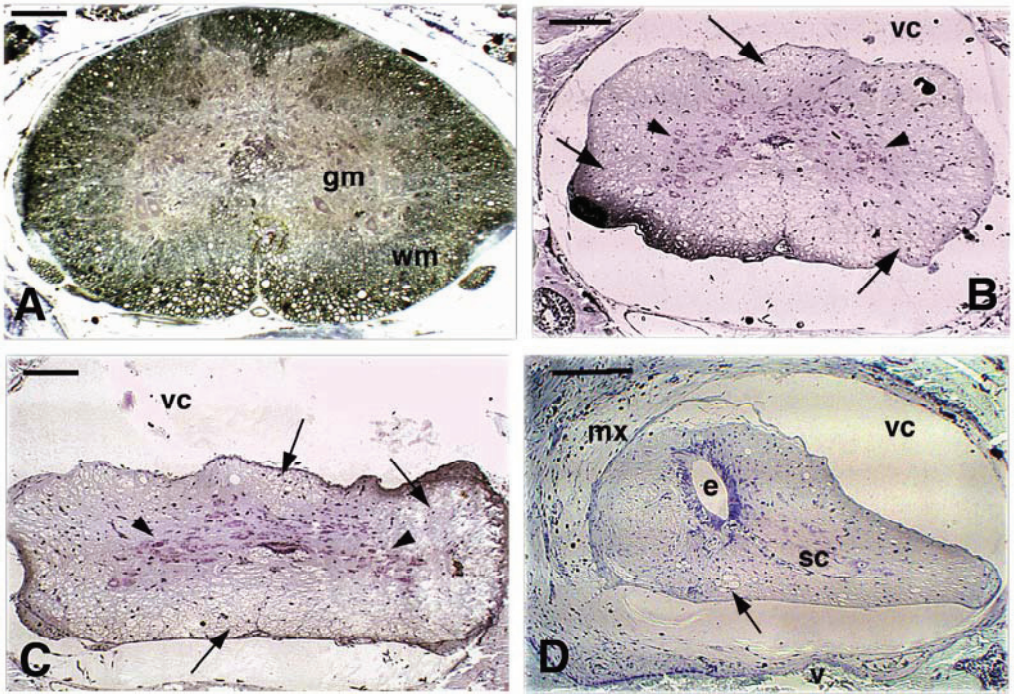
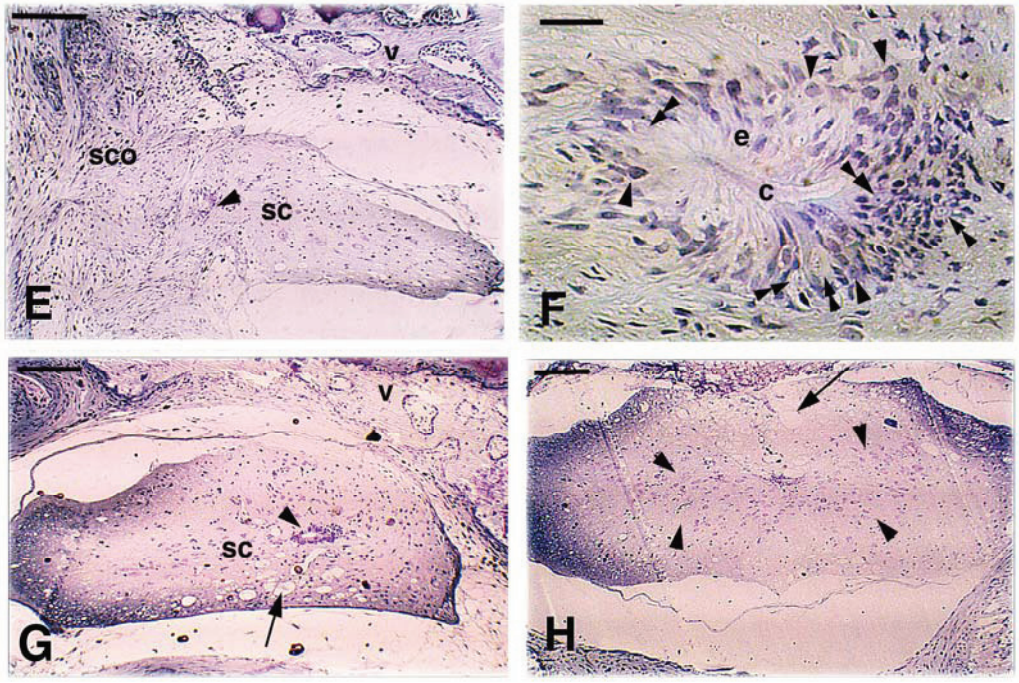


**Figure 6.** *Cont.*




The analysis of fluorescent-Dil tracer distribution in case #1 showed that the tracer was mainly applied and adsorbed in the dorsal-lateral right side and in the ventral-medial right side of the distal spinal cord, localized caudally to the bridge tissue (Figure 7A). This area included mainly white matter and the grey matter of the right dorsal horn. This labeled area became more diffuse moving toward the narrower bridge region where some intensely labeled fibers were seen in the central part of the SC (Figure 7B–D). After moving 2–3 mm rostral to reach the proximal spinal cord stump, numerous fluorescent fibers were seen, and they were mainly localized in the medial ventral part of the white matter (Figure 7E). Sparse small or large neurons present in various areas of the grey matter of the proximal spinal cord were also retrograde-labeled with the fluorescent Dil (Figure 7E–H). No fluorescent labeled, large neurons in the ventral grey were seen.

The microscopic analysis of distal-proximal cross-sections from case # 2 showed a more diffuse Dil-fluorescence over most grey and white matter in the distal spinal cord (the region of application of the tracer), although a much higher fluorescent area was present in the left dorsal white matter (Figure 8A,B). Moving proximally toward the transected region, the collected cross-sections showed few labeled fibers present in the narrow bridge spinal cord, some especially intense in the dorsal part (Figure 8C). In more proximally collected sections, sparse fluorescent neurons were observed in the grey matter of the proximal stump of the spinal cord, and also in the proximal spinal cord located at 5–7 mm form the point of application of Dil (Figure 8D–G). These small to elongated neurons were mainly present in the intermediate grey matter, and no Dil-labeled motor-neurons were seen.

Also the other two cases showed similar features, indicating the presence of Dil-labeled fibers within the bridge. The study on the distribution of the retrograde Dil-labeled neurons in the grey matter and of the axons in the white matter of the proximal spinal cord stump in all four cases is summarized in Figure 9.

**Figure 7.** Representative proximal-distal cross-sections of the spinal cord in case 1 experiment (thin bridge tissues with modest recovery) after DiI application. A, section close to the point of DiI application (arrows on most labeled areas). The arrowhead points the position of the central ependymal canal. gm, grey matter. Scale bar, 0.2 mm B, more rostral section within the caudal SC stump (the arrow shows the region with higher tracer-load). Asterisks indicate missing areas of the SC (artifact derived from the vibratome sectioning). Scale bar, 0.15 mm. C, further rostral region preceding the bridge between proximal and distal SC stumps. The arrow indicates the most labeled area. The asterisk indicates some missing tissue due to sectioning. Scale bar, 0.1 mm. D, narrow diameter of the bridge region showing sparse labeled axons (arrowhead). Scale bar, 0.1 mm. E, rostral spinal cord past the bridge area showing a large labeled region in the white matter (arrow) and some cells (arrowheads) in the grey matter. gm, grey matter. Scale bar, 0.15 mm. F, rostralmost region of the SC with labeling in the white matter (arrow) and inside cell bodies (arrowheads) in the grey matter (gm). Scale bar, 0.15 mm. G, detail of a labeled neuron and its likely axonal elongation. Scale bar, 10 μm. H, other two small neurons present in the intermediate region of the grey matter. Scale bar, 20 μm.

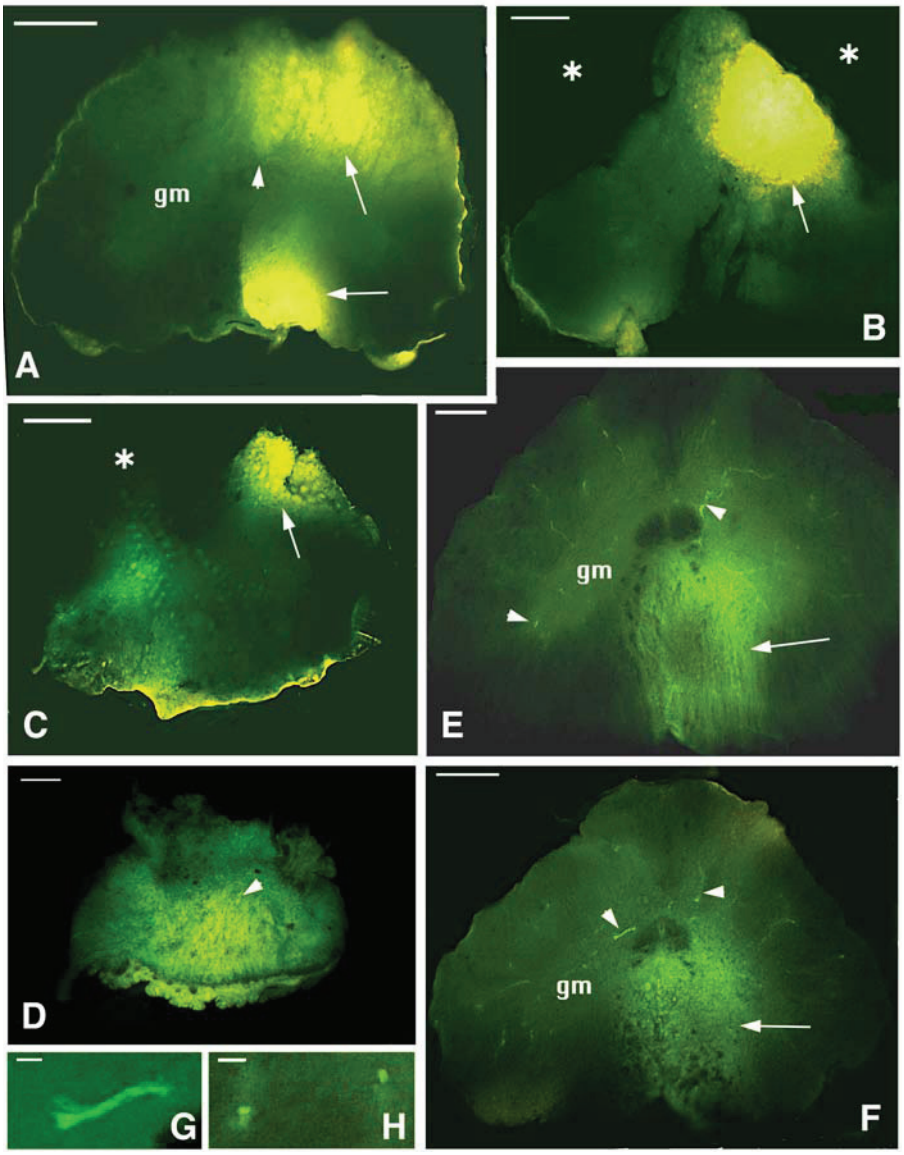
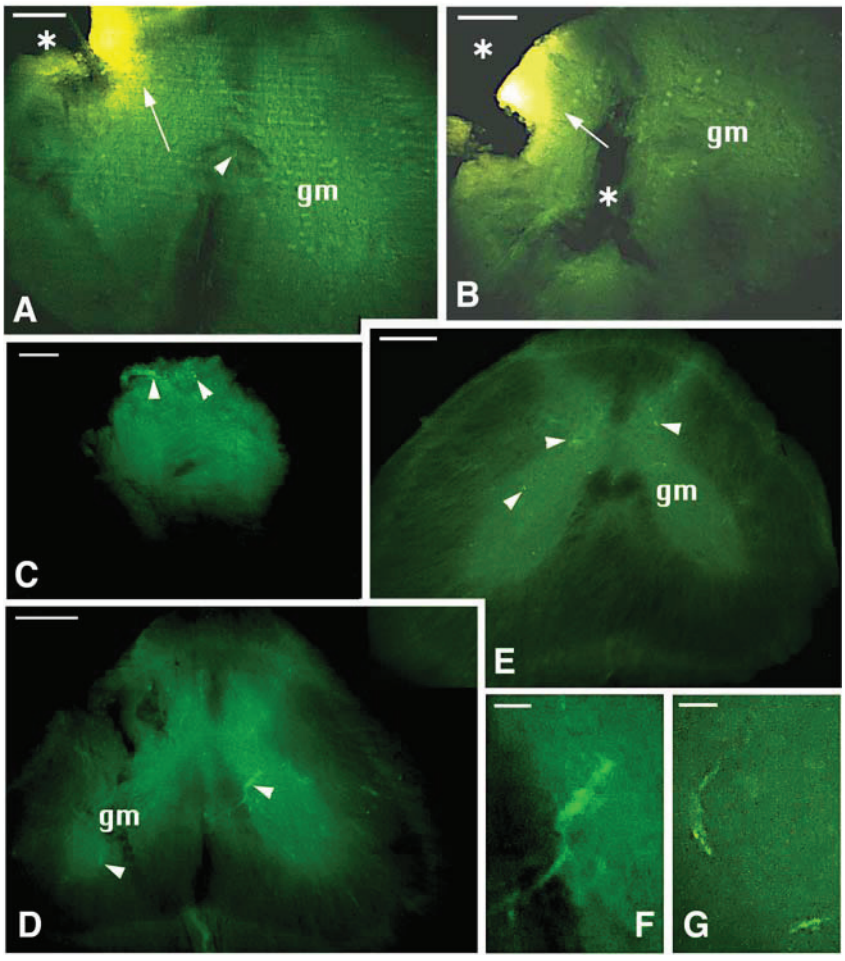


**Figure 8.** Proximal-distal representative cross-sections of the SC in case 2 (completely transected with poor recovery) after DiI application. **A**, caudalmost area located near the point of application of the tracer (the arrow points to the region that has incorporated most of the tracer). The asterisk indicates that some tissue is missing due to the sectioning. The arrowhead points to the central canal. gm, grey matter. Scale bar, 0.1 mm. **B**, more rostral section of the SC near the bridge region (the arrow indicates the most labeled area). Asterisks point to regions with missing tissue due to the sectioning. gm, grey matter. Scale bar, 0.1 mm. **C**, narrow spinal cord in the bridge area. The arrowheads indicate few axons containing higher levels of the tracer. Scale bar, 0.1 mm. **D**, cross section of the rostral spinal cord stump past the bridge region showing some labeled neurons (arrowheads) in the grey matter (gm). Scale bar, 0.15 mm. **E**, a rostral most section showing most labeled neurons (arrowheads) in the medial-upper part of the grey matter (gm). Scale bar, 0.15 mm. **F**, detail on a labeled neuron in the medial ventral grey matter. Scale bar, 10 μm. **G**, detail of labeled neurons localized in intermediate grey matter area. Scale bar, 10 μm.




## 4. Discussion

### *4.1. Recovery Behavior and Possible Reactivation of the Local Spinal Motor Generator*

The present, qualitative study confirms and increase the information on the general recovery behaviors previously described in lizards with lumbar and thoracic spinal cord transection [11,18]. In

these studies on lizards with complete or partially transected spinal cord, the animals begun to move their limbs again at 20–30 days post-transection, after the initial 10–15 days of complete paralysis. It is not clear from the above experiments whether the dorsal part degenerated while the ventral or motor region of the cord was still connecting the proximal with the distal stumps of the spinal cord, or whether this was due to regeneration of nerve tissue from the two SC stumps. In the above studies on recovery function in lizards [11,18], it is not clear whether the tail was also regenerating during the recovery period of the spinal cord. Since no mention was made on tail regeneration we assume that the reported recovery was not associated to tail regeneration but that the lizards conserved their original tails, suggesting that tail regeneration does not influence the recovery. This previous qualitative study [11] however did not determine whether new neurons were present in the bridge tissue, while the present study suggests that some neurons are reformed within the bridge, and the formation of nerve connections between the distal stumps and some neurons localized in the proximal stump.

In the present study, it is not definitely shown whether the presence of a regenerating tail influences the recovery of the spinal cord, and this point remains to be clarified in future experiments. The transected lumbar spinal cord was located at about 1.5–2 cm from the regenerating tail, which generally reformed a new tail. Whether the regenerating tissue of the tail may have influenced the growth of the lumbar spinal nerves through a long-distance (1–2 cm) to innervate the new tail is therefore not known. Also, it remains to be studied whether the connective and muscle tissues that regenerated inside the new tail may have somehow influenced the regeneration of axons across the bridge, enhancing the sprouting of axons from local interneurons like in the tail spinal cord [10]. The unique condition found in lizards, namely the vicinity of the injured lumbar spinal cord to the forming connective and muscle tissues of the regenerating tail, could represent an advantageous model for future studies to analyze the diffusion of growing factors capable to influence the regeneration of long supraspinal connections.

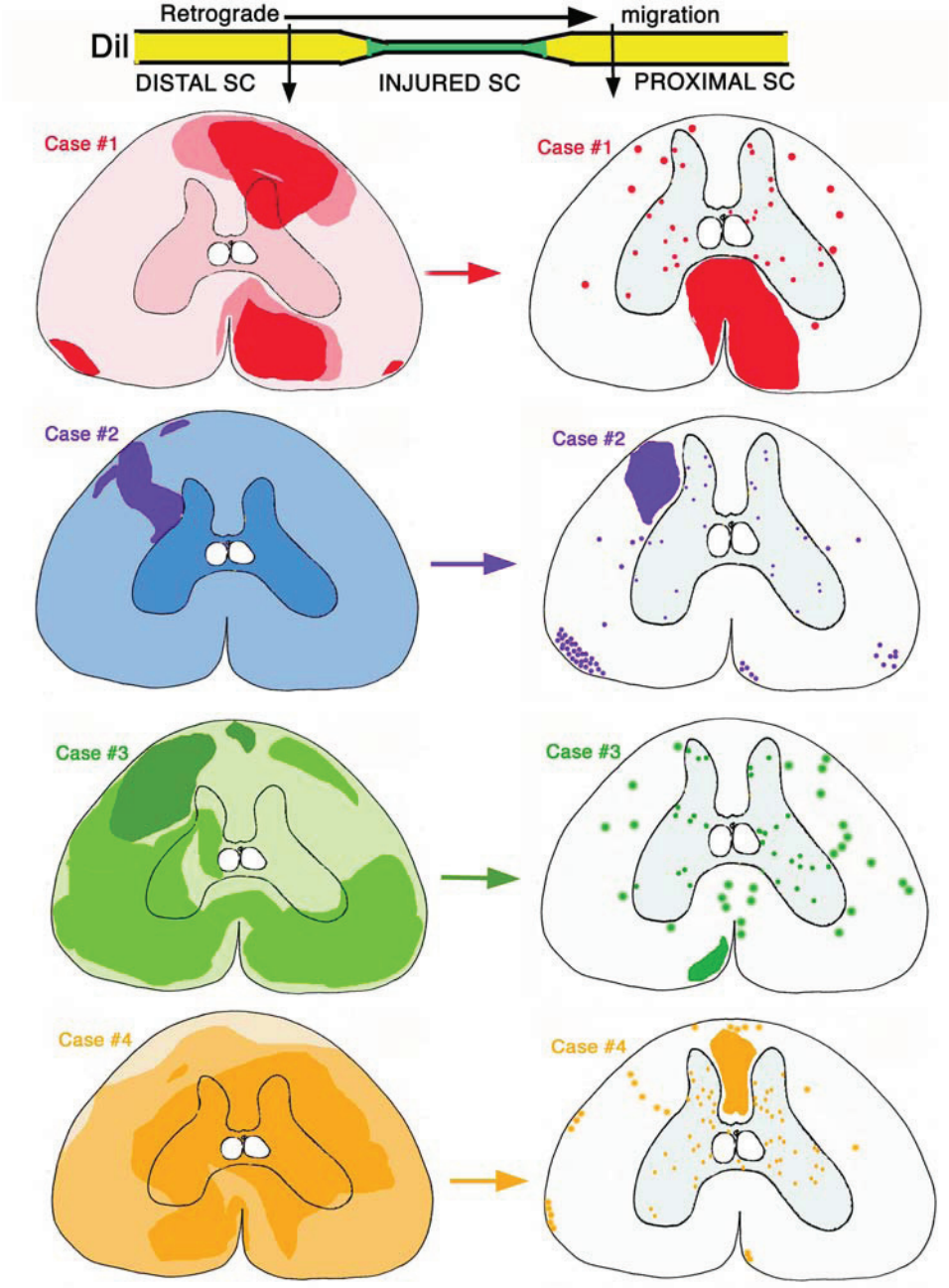
The variable recovery of stepping movements in the hind limbs in most operated lizards suggests that the retrograde-labeled neurons send their regenerated axons across the bridge within a 20–30 days period after the lesion. That the limbs movements were not simple spinal reflexes was also indicated from the fact that the lizards voluntary stepped away after a tap noise was applied to the wall of the cages or a hand was introduced in the cage (escaping reaction).

The recorded retrograde-labeled small neurons in our four Dil-tracing cases, likely represent interneurons located within sensory and motor areas of the gray matter of the spinal cord, while motor neurons were not labeled in these experiments. From the number of recorded retrograde-labeled neurons (Figure 9) it appears that case #2 (with poor functional recovery) has the lower number of retrograde-labeled neurons in the grey matter while the other three cases (#1, 2 and 4) with more functional recovery (modest-good) possess more retrograde-labeled neurons, suggesting a correlation between the number of labeled neurons and functional recovery. The lowering or complete disappearing of motor neurons in the distal part of the spinal cord indicates that the transection in most cases affected their innervation and/or the blood vessel sustaining their relatively high metabolism in comparison to smaller neurons. The present observations also suggest that the retrograde-labeled small neurons may belong to an intraspinal "central motor locomotor system" in lizard that is believed to be present in vertebrates, from fish to mammals [20–23] (Puskar and Antal). However, the details on the

characteristics and specific circuits of the neurons reconnecting the two stumps of the injured spinal cord need more specific studies.

A similar, partial recovery of the motility of hind limbs has been recently described for turtles [13,14] indicating that some of the motor circuits present in the spinal cord of these reptiles are capable of reactivation after lesion. Based on these observations it appears that reptiles represent a useful experimental model for studies on spinal cord regeneration in amniotes.

**Figure 9.** Schematic drawing showing the total of the labeling results (neurons within the grey matter and labeled fibers in the white matter) in the four cases studied. On the left is shown the distribution of the tracer in the distal spinal cord where the tracer was placed, and the color intensity roughly indicate the intensity of the labeling. On the right column are indicated the position of all the counted labeled neurons (smaller dots within the grey matter) and axons-fibers (larger dots within the white matter).



*4.2. Histological Aspects of Recovery*

The permanence in the distal spinal cord past the bridge of large part of the grey matter confirms the anatomical and physiological autonomy previously observed for the spinal cord in lizards [11,18], although numerous axons in the white matter degenerate. The presence of dislocated vertebrae that partially occluded the vertebral canal might also have contributed to differences in recovery capability. The observed loss of motor-neurons, especially in the distal spinal cord stump, is probably correlated to the poor or absent strength of their hind limbs for walking.

The numerous small glial cells seen in the bridge spinal cord indicate that the trauma has stimulated the local proliferation of these cells together fibroblasts of the meninges with which the glial tissue is often in continuity when the lesion has damaged extensive areas of the meninges. Despite the scarring connective, several axons appear to cross the bridge between the two stumps of the transected spinal cord, reconnecting anatomically and functionally the interrupted spinal cord. Whether the cells present in the bridge spinal cord represent astrocytes, oligodendrocytes, microglia, or even include some new neurons generated from the stratified ependyma, will be specifically analyzed by an ultrastructural and immunocytochemical analysis. However a preliminary ultrastructural report indicates that axonal regeneration takes place in the bridge region of the transected spinal cord [19].

In the caudal spinal cord of lizards, initial microscopic and ultrastructural studies indicated that no neurogenesis was present [9,10]. However, other microscopic, ultrastructural, autoradiographical, and immunocytochemical studies showed that few and specialized neurons were instead regenerated within the tail SC [10,12,16,17]. Whether the new cells present in close association with the ependymal epithelium of the injured lumbar SC of lizards noted in the present study are neural or glial cells awaits further ultrastructural and immunocytochemical studies.

## 5. Conclusions

The present study indicates that a limited but functionally important spinal cord recovery is present in lizards after loss of large areas of the lumbar spinal cord. The axonal and the scarce neuronal recovery of the lumbar spinal cord allow the animals to move their hind limbs, although their strength for locomotion appears largely lost. Whether the general disappearing of motor neurons in the distal spinal cord are at the origin of the loss of stimulation for hind limb strength is not known but this point deserves a further analysis.

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## Author Contributions

Planned, conducted technically, discussed, and largely self-supported financially.

## Conflict of Interests

The author declares no conflict of interest.

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