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Ultrastructural Observations on Lumbar Spinal Cord Recovery After Lesion in Lizard Indicates Axonal Regeneration and Neurogenesis

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Abstract

After lumbar spinal cord transection, lizards recover some un-coordinated movements of the hind limbs including some walking ability. The transected spinal cord was examined using electron microscopy to study the degree of regeneration. A bridge tissue located between the two proximal and distal ends of the transected spinal cord, representing the regenerated cord, was found at 29-45 days post-lesion. The ependyma formed ampullar enlargements but no continuous central canal between the two stumps was re-established. Some cerebro-spinal fluid contacting neurons and peri-ependymal small neurons contacted by few synaptic boutons were found. Pale cells of astrocyte type were also identified. Numerous unmyelinated and sparse myelinated axons in the bridge appear connected to ependymal cells and oligodendrocytes. The origin of these axons remains unknown but previous studies have indicated that at least some of them are derived from interneurons, including those located around the central canal and in the dorsal grey matter of the proximal spinal cord stump. This limited regeneration can explain the recovery of part of the motor activity of the hind limbs in these lizards, possibly through the re-connection across the bridge of the intrinsic circuit of the central locomotory pattern generator.

Keywords: lizard, spinal cord, lumbar transection, regeneration, microscopy, ultrastructure

1. Introduction

The lesion of the spinal cord (SC) in homeothermic amniotes (birds and mammals) determines a permanent paralysis with the formation of connective scar in the point of lesion of the SC (Schwabb and Bertholdi, 1996; Brodgen, 2003). This is not the case for lower vertebrates such as the cyclostomes, teleost fish, and aquatic salamanders that, after an initial paralysis recover a variable degree of motility (Piatt, 1955; Butler and Ward, 1967; Holder and Clarke, 1988; Bernstein, 1988; Davis et al., 1989, 1990; Ferretti et al., 2003). The recovery generally occurs without the reconstitution of the anatomical structures (grey and white matter) present in the original SC before the injury. In fact, both cellular and fibrous regeneration result in a much simpler anatomical organization and connections of the regenerated SC with respect to the initial condition (Stensaas, 1983; Davis et al., 1989, 1990).

Among the reptiles, a class that includes the basal amniotes from which both birds and mammals originated, the regeneration of the SC is poorly known. Lizards can form a simplified spinal cord in the regenerating tail where few axons, glial and rare small neurons are regenerated (Simpson, 1968; Egar et al., 1970; Alibardi, 1990-91, 1993; Alibardi et al., 1993; Zhou et al., 2012). Recent studies have shown that the transected thoracic spinal cord in turtles has a limited but remarkable functional and anatomical regeneration (Reherman et al., 2009, 2011), similarly to previous observations conducted on lizards (Furieri 1957; Raffaelli and Palladini, 1969; Alibardi, 2010).

As regard the regeneration in more rostral parts of the SC some conflicting results have been obtained after lesion of the thoracic or of the lumbar spinal cord in different species of lizards. In fact, the histological analysis of transected lumbar SC of the lizard *Podarcis muralis* that recovered walking at 20-30 days post-injury indicated axon regeneration capable to reconnect the distal with the proximal sides of the lumbar SC (Raffaelli and Palladini, 1969). Neither a partial recovery nor regeneration of the thoracic SC were instead observed in the lizard *Anolis carolinensis* after thoracic or lumbar lesions (Simpson, 1983; Simpson and Duffy 1994). In order to clarify this important issue on the possibility of SC regeneration in amniotes, a recent study on lumbar SC lesions in lizard has shown that *Podarcis muralis* can indeed partially recover hind limb movements and show clear signs of axonal and also cellular regeneration (Alibardi, 2010, and unpublished observations).

The cytological details of the process of regeneration however need the use of the electron microscope in order to clarify the nature of the regenerated cells (glial or/and neural), and unequivocally identify regenerating axons across the lesion SC. In order to fulfill the above goal, the present ultrastructural study is an extension of the previous tract-tracing study, and shows that the process of lumbar SC recovery in lizard determines the formation of new glial cells, some neurons and axons in the bridge area between the proximal and distal stumps of the spinal cord lesion. The study indicates that the lizard model of spinal cord lesion is ideal for increasing the knowledge on the mechanisms operating during spinal cord regeneration in amniotes.

2. Materials and Methods

Adult lizards (*Podarcis muralis*) of both sexes were utilized in the present study, and all the surgical procedures utilized in the study followed the regulations on animal care and experimentation procedures under the under the Italian and European Guidelines (art. 5, DL 116/92).

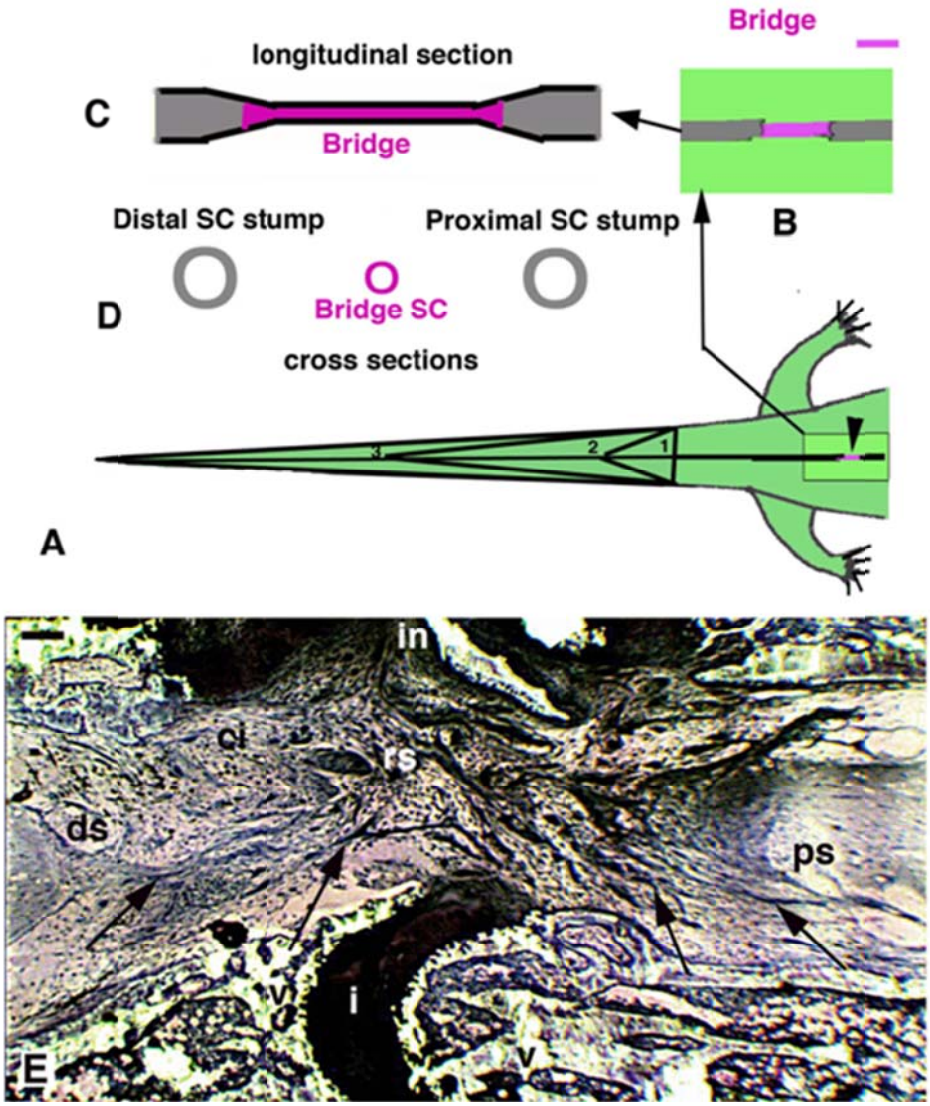

Figure 1. Schematic drawing describing the removal of a small segment of the lumbar spinal cord (arrowhead in **A**) connected with that of the regenerating tail (1, 2, 3 represent three progressive stages of tail regeneration), the regeneration of a bridge but thinner SC at 29-45 days post-amputation (violet color in **B**), and the modality of sectioning of this region in longitudinal (**C**) or in cross section (**D**) (see text). **E**, histological aspects of longitudinal sections between the proximal and the ventral spinal cord stumps showing some nerve fascicles (arrows) crossing the bridge. Bar, 0.1 mm. **Legend**s: ci, cicatrix tissue; ds, distal stump of the spinal cord; i, inter-vertebral cartilage; in, injured neural arch; ps, proximal stump of the spinal cord; rs, regenerated spinal cord (bridge)

Eight individuals with transected lumbar spinal cord, previously utilized for the analysis of behavioral recovery and histology at 29, 36 and 45 post-lesion were also utilized for the present electron microscopic study (Alibardi, 2010, 2014). The progressive time-points were selected to detect possible variations of cell differentiation in the regenerated lumbar spinal cord since previous studies on the caudal spinal cord indicated that glial cells and neurons differentiate within 20-30 days from ependymal precursors in regenerating tails of 30-40 days (Alibardi, 1993, 2010). Briefly, the animals were anesthetized with ethylic ether and the skin, dorsal and inter-vertebral muscles were sectioned until the vertebral column was visible. Using a sharp scissor a quick snap-cut was made between the 3rd or the 4th lumbar vertebra from the pelvic girdle, as schematically shown in Figure 1 A-B. Following SC transection the tail started to oscillate for less than a minute, bleeding was blotted and stopped rapidly. Vertebrae were re-aligned and the severed dorsal muscles were recomposed over the vertebrae. A cicatrizing and antibiotic powder (Cicatrene, Welcome Italia, Pomezia-Rome) helped in sealing the wound and stopping bleeding while the skin was recomposed over the underlie tissues. Since some animals dropped their tail after the transection we wanted to keep all the lizards in the same condition of amputated tails (Figure 1 A 1-3). This also allowed to determine whether tail regeneration during the entire span of the experiment was influences by the close spinal cord transection.

Samples of regenerating spinal cord were collected at 29 (n = 3), 36 (n = 3), and 45 (n = 2) days post-injury, and fixed in 3% glutaraldehyde in 0.1 M Phosphate buffer at pH 7.4 for about 48 hours at 4°C. The long fixation period was chosen in order to fix en-block the large pieces of lesion area, pieces that measured about 5-6 mm in length and were 1-2 mm wide. During fixation large part of the vertebrae were cut open to allow the rapid penetration of the fixative into the SC. The tissues were rinsed in buffer for about 2 hours, and post-fixed for 3 hours 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 hours in pure Araldite resin before curing the blocks for 3 days at 60°C. This procedure aimed to infiltrate all the very heterogenous tissues of the sampled material that also included the bone tissue of the vertebrae.

The embedded tissues were sectioned with an ultramicrotome at 1-3 μm in thickness in the longitudinal plane (1 case from 29 days and 1 case from 36 days post-lesion, Figure 1 C) and in cross-sections (2 cases from 29 days, 2 cases from 36 days, and 1 cases from 45 days post-lesions, Figure 1 D). 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 cross plane, the proximal (rostral) stump of the SC was sectioned proceeding toward the transected area (bridge), 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 (bridge) between the proximal and distal SC stump, about 5 sections were collected every 30-40 μm. In areas of interest in the bridge, also 60-90 nm thick sections of the entire SC were collected on copper grids, stained in uranyl acetate and lead citrate according to standard methods, and observed under a Zeiss 10C/CR transmission electron microscope.

3. Results

3.1 Light Microscopy

At 29 and 36 days post-lesion, the repaired spinal cord in the longitudinally sectioned samples showed a larger proximal and a distal stump connected by a narrow bridge tissue (Figure 1 C, E). The latter consisted of sparse and irregular bundles of nerve fibers (arrows in Figure 1 E) embedded within a connective tissue mixed with glial cells connecting the two stumps of the original spinal cord. In case of massive lesions to the meninges and neural arch of the vertebrae, also a scarring connective tissue filled up the vertebral canal around the bridge tissue, and contacting the glial bridge tissue.

The study of cross-sections at 29 days post-lesion (Figure 2), and 36 and 45 days post-lesion showed similar features of the injured stumps and the bridge tissue. The most proximal sections, taken at about 1-2 mm rostral from the bridge tissue, showed a regular H-shaped grey matter surrounded by a richly myelinated white matter (Figure 2 A). Moving toward the bridge the spinal cord became shrunken, many myelinated axons disappeared and in various regions of the white matter vesicular areas of degenerated axons were seen (Figure 2 B, C). In the grey matter neurons were well visible for their granular cytoplasm rich in Nissl bodies while the central canal appeared enlarged in comparison to the more proximal sections. In more close sections to the site of transection (Figure 3 C), the ependymal canal was even more enlarged and the epithelium was formed of elongated ependymal cells. A further broadening of the ependymal canal corresponding to the dilated ampullae observed in longitudinal section, was observed in more caudal sections of the bridge while the grey matter was largely

disappeared in this region (Figure 3 D). The observation at higher magnification showed that the ependyma was stratified and among ependymal cells some round cells indicated that either glial or neural elements were present (Figure 3 E), as it was later confirmed by the electron microscope.

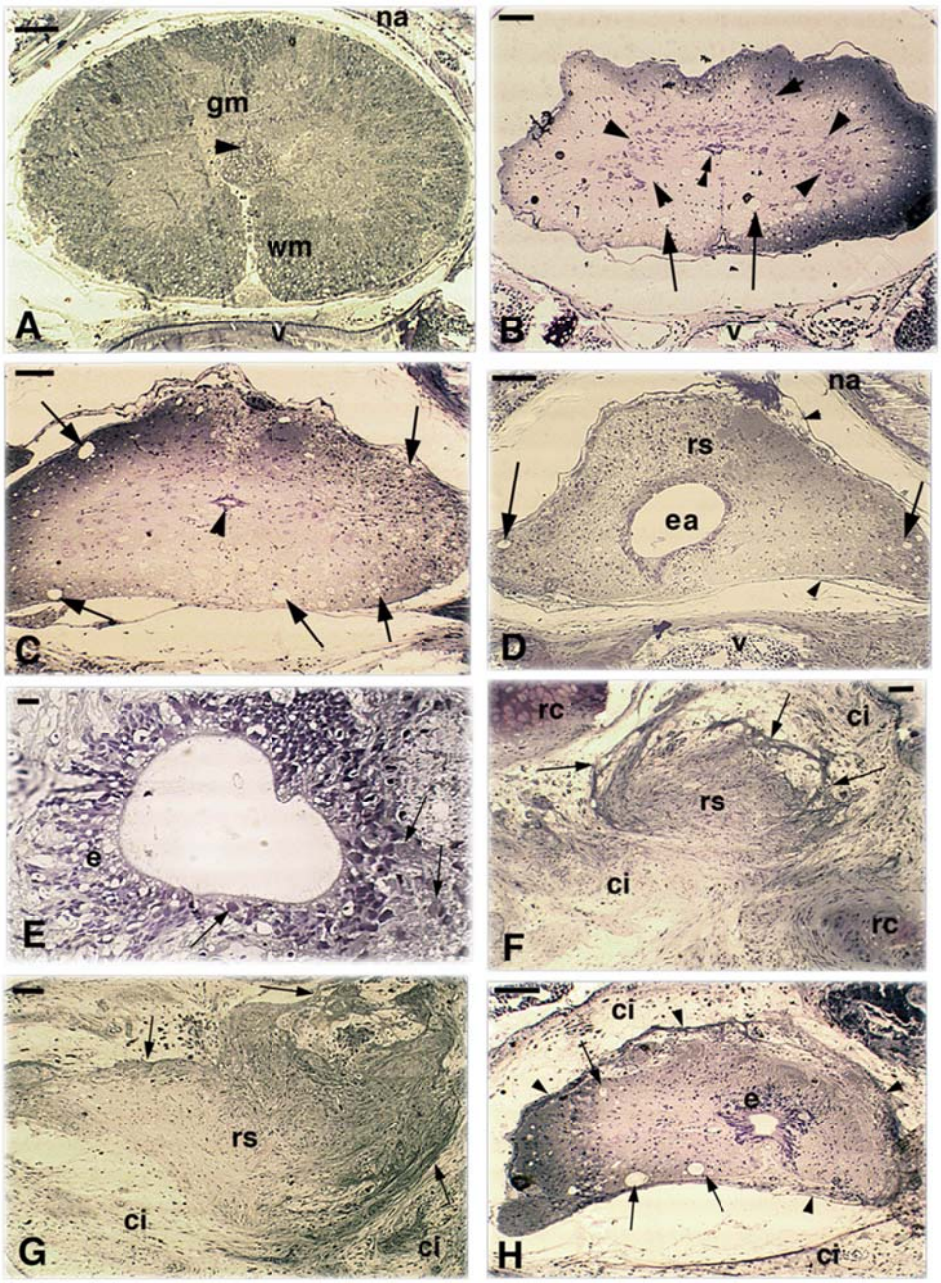

Figure 2. Representative rostral-caudal sequence of cross sections of 29 days post-lesion SC from the proximal stump of the SC (A-E), the bridge (F, G) and distal stump of the SC (H). Toluidine blue staining. **A**, normal aspect of proximal SC (the arrowhead points to the small ependymal canal). Bar, 0.1 mm. **B**, reduction in size of SC closer to the point of lesion. The grey matter is well visible (arrowheads) and in the white matter numerous myelinated axons are degenerating (arrows). The double arrowheads points to the slightly enlarged ependymal canal. Bar, 0. 1 mm. **C**, a further section closer to the bridge tissue shows the loss of the H shape of the grey matter, reduction of white matter containing numerous degenerating axons (arrows), and the enlargement of the ependymal canal (arrowhead). Bar, 0.1 mm. **D**, a distal level of the rostral SC stump showing the broad enlargement of the ependymal canal (ampulla), the reduction of the diameter of the SC and the loss of the organization in grey and white matter where numerous degenerating axons are seen (arrows). Numerous small (glial) cells are evenly distributed in this section of regenerated spinal cord. The arrowheads indicate the meninx. Bar, 0.1 mm. **E**, detail showing the stratification of the ependyma in the ampulla. Among ependymal cells some neural or glial cells are present (arrows). Bar, 20 µm. **F**, bridge tissue between the two SC stumps composed of glial-tissue and some nerves, and surrounded by scarring connective tissue in continuity with the meninges (arrows). Bar, 50 µm. **G**, more caudal section of the bridge tissue still surrounded by scarring connective in continuity with meningeal membrane (arrows). Bar, 100 µm. **H**, caudal SC stump past the bridge region showing a still enlarged ependymal canal and numerous large vacuolated spaces from degenerated axons (arrows). arrowheads indicate the meninges. Bar, 0.1 mm. **Legends**: b, bridge tissue; ci. cicatricial (scarring) connective tissue; e, epednymal epithelium; ea, ependymal ampulla; gm, grey matter; na, neural arch of the vertebra; rc, regenerated cartilage; rs, regenerated cord (bridge); v, vertebral bone; wm, white matter

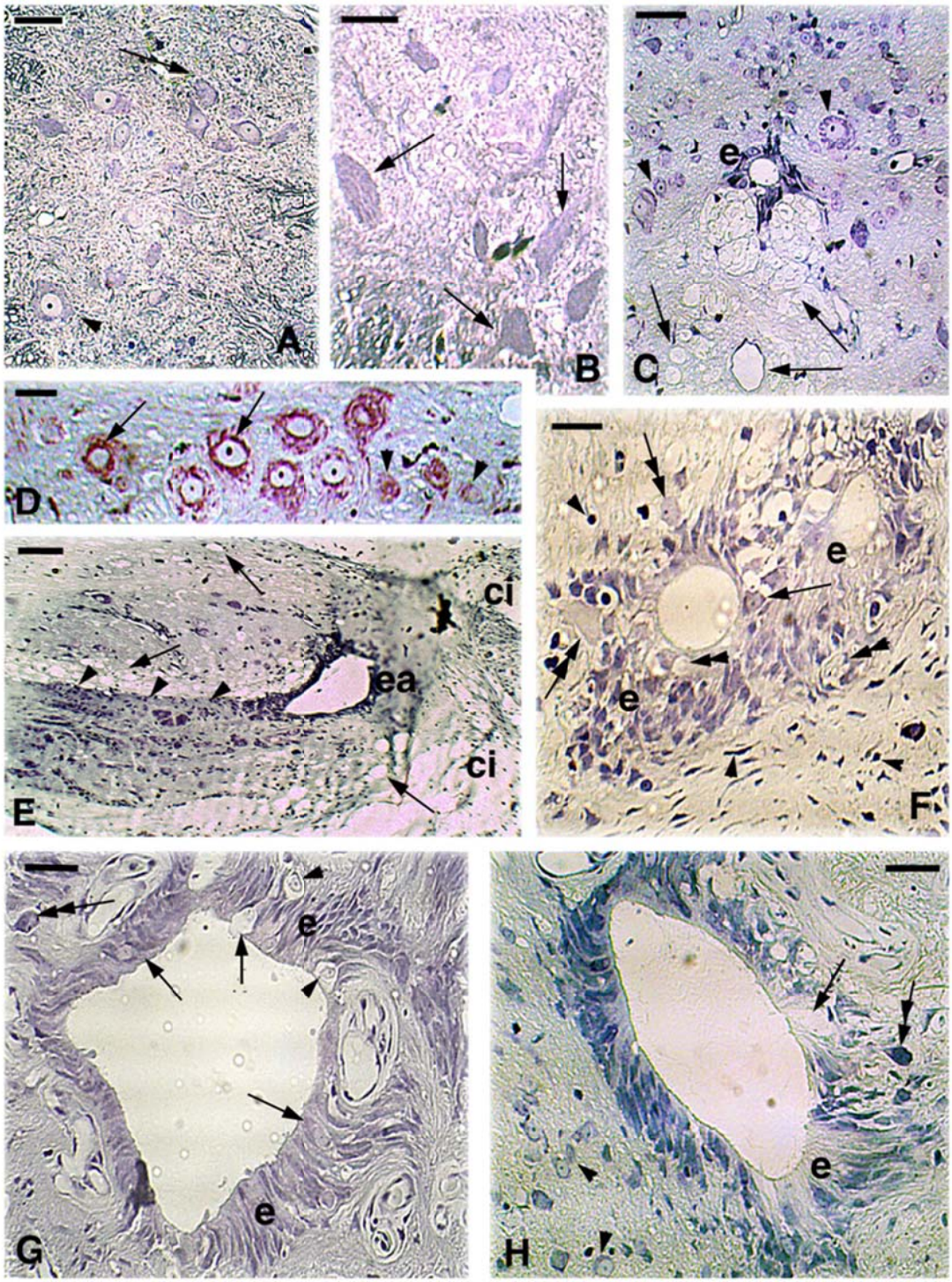

Figure 3. Cytological details showing cells in the rostral SC (pre-lesion, **A**-**G**) and caudal SC (post-lesion, **H**) at 29 days post-lesion. Toluidine blue staining. **A**, normal aspect of neurons (arrow, small-medium size; arrowhead, large size) and myelin in a rostral level of the SC stump (about 1.5 mm from the bridge region). Bar, 15 µm. **B**, detail on the even staining of cell bodies of motorneurons (arrows) in the ventral horn of the proximal SC. Bar, 15 µm. **C**, other detail of some medium-large neurons with granulated Nissl material (arrows). Bar, 10 µm. **D**, central canal (slightly enlarged), degenerating vacuolated axons (arrows) in the longitudinal medial fasciculus in proximal SC close to the bridge region. Large and small neurons (arrowheads) show a granulated cytoplasm (distinct Nissl material). Bar, 20 µm. **E**, detail of the enlarged terminal ependymal ampulla in the rostral SC stump where sparse degenerating axons are seen (arrows). Numerous neurons are still present in the stump around the central epithelium of the ependyma (arrowheads). Bar, 50 µm. **F**, cross section detailing the stratification of the ependyma in the proximal SC near the bridge region. A neuronal-like cell is seen (arrow). The double arrows indicate neural cells outside the ependyma. Arrowheads indicate smaller glial cells. Double arrowheads indicate possible astroglial cells. Bar, 15 µm. **G**, other detail on an enlarged central canal showing elongated ependymal cells or tanicytes. Arrows points to possible Cerebro Spinal Fluid Contacting Neurons the arrowhead to an astrocyte, and the double arrow a neuronal body external to the ependyma. Bar, 15 µm. **H**, detail of enlarged ependymal ampulla present in the caudal SC stump past the bridge region. A likely Cerebro Spinal Fluid Contacting Neuron (arrow) and an external neuronal-like cells (doublearrows) are seen. Other small neurons are present in the surrounding nervous tissue (arrowheads). Bar, 15 µm. **Legends**: ci, cicatrix, scarring tissue; e, ependymal epithelium; ea, ependymal ampulla

In a more caudal section across the bridge tissue, corresponding to about the central part of the regenerated cord, the ependymal canal disappeared and a compact mass of tissue made of numerous, small cells of glial and connective nature was observed (Figure 2 F). This part of the regenerated spinal cord or bridge was in contact with the fibrous meninges (arrows in Figure 2 F, G). Around the meninges, a cicatrix connective occupied most of the vertebral canal, delimited by bony parts of vertebrae and by regenerated cartilaginous tissue (Figure 2 F). Moving toward the stump of the distal spinal cord, caudally to the bridge tissue, the enlarged and stratified ependymal ampulla re-appeared (Figure 2 H). In more distal sections also the grey matter with numerous neurons was observed although the large motor-neurons were generally absent and numerous axons in the white matter appeared vacuolated (data not shown).

The observation at higher magnification of the cells present in the rostral and caudal spinal cord stumps revealed that the neurons located in the rostral stump showed an evenly stained cytoplasm with denser Nissl areas (Figure 3 A, B) while those close to the bridge featured a cytoplasm with clumps of stained Nissl material and much less stained cytoplasmic areas (Figure 3 C-D). Also numerous vesicles representing degenerated axons were seen in the white matter surrounding the enlarged ependymal ampulla (Figure 3 E).

The cell composition at 29, 36 and 45 days post-lesion was similar. Cells in the bridge were small, with round or elongated but narrow nuclei, representing glial cells that had probably intensely proliferated after the lesion (Figure 3 F). In the ependymal epithelium however, among the elongated tanicytes, few basophilic or other pale cells with round nuclei and large nucleoli were seen (arrows or double arrows in Fig 3 F-H) resembling small neural cells or pale astrocytes (arrowheads or double arrowheads in Figures 3 F-H). Some pale cells even contacted the central canal suggesting that they represent cerebro-spinal fluid contacting neurons, as later confirmed with the electron microscope (triple arrowheads in Figure 3 G-H). Other small neural cells appeared external to the ependymal epithelium, suggesting they correspond to cells migrating outside the ependyma (double arrows in Figure 3 F-H). These neural cells were very similar to the small neurons surrounding the central canal (arrowheads in Figure 3 H), and possessed a pale nucleus and one tow large nucleoli.

3.2 Ultrastructure

Despite the fixation adopted in the present study for large pieces of the spinal cord was not optimal for the ultrastructural details, it was however possible to identify in the bridge tissue not only truly degenerated cells and nerves, but also neural or glial cells including their dendrites and axons still in course of regeneration. The electron microscopic analysis of cells and axons in the proximal and distal spinal cord stumps and in the bridge at 29, 30, and 45 days after lesion showed similar aspects, and we here show some examples in the 29 post-lesion sample (Figures 4-8).

The examination of the enlarged central canal of the ependymal ampulla at 29 days after spinal cord transection showed elongated ependymal cells forming a stratified epithelium (Figure 4 A). Among ependymal cells, typical pale elements with a pear-shape, secretory vesicles and euchromatic nucleus were identified as Cerebro-spinal Fluid Contacting Neurons (Figure 4 A, B). In fact, their apical cytoplasm contacting the central canal showed a number of stereocilia, the cytological landmark for these cells, sparse among the microvilli of ependymal cells (Figure 4 C). The examination of other pale cells of 8-12 μm in diameter, localized in the peripheral region around the ependyma (see arrows in Figures 2 E and 3F, and double arrows in Figure 3 G, H) showed typical characteristics for differentiating neurons: roundish and pale cytoplasm, euchromatic nucleus with a large nucleolus, formation of stacks of rough endoplasmic reticulum, increase in the number of mitochondria, formation of a pale elongation recalling an elongating axon (Figure 5 A, B, 6 A). The study at higher magnification showed sparse synaptic boutons with pale or dense vesicles contacting or passing close to the plasma membrane of these cells (Figure 6 B, C).

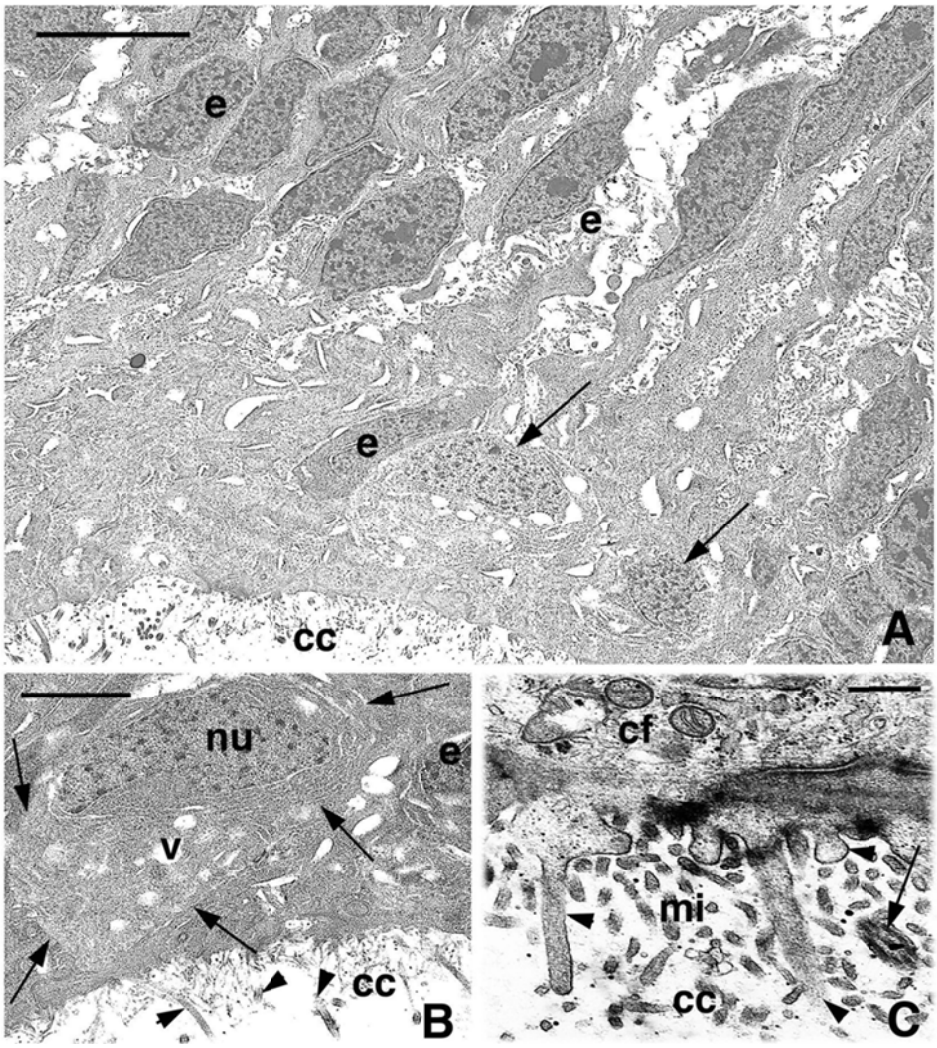

Figure 4. Ultrastructural details of the central canal of enlarged ependymal ampulla at 29 days post-lesion. **A**, elongated and stratified ependymal cells and two neuronal cells (arrows) located near the central canal. Bar, 5 μm. **B**, detail on a cerebro-spinal fluid contacting neuron (arrows indicate the outline). The arrowheads indicate the stereocilia inside the central canal. Bar, 2 μm. **C**, detail of the electron-pale cytoplasm of a cerebro-spinal contacting neuron sending stereocilia (arrowheads) inside the central canal. The arrow indicates a true cilium. Bar, 0.5 μm. **Legends**: cc, central canal; cf, luminal cytoplasm of a cerebro-spinal fluid contacting neuron; e, elongated and narrow ependymal cells (tanicytes); mi, microvilli of ependymal cells; n, neuron (cerebro-spinal fluid contacting neuron); nu, nucleus; v, secretory vesicles

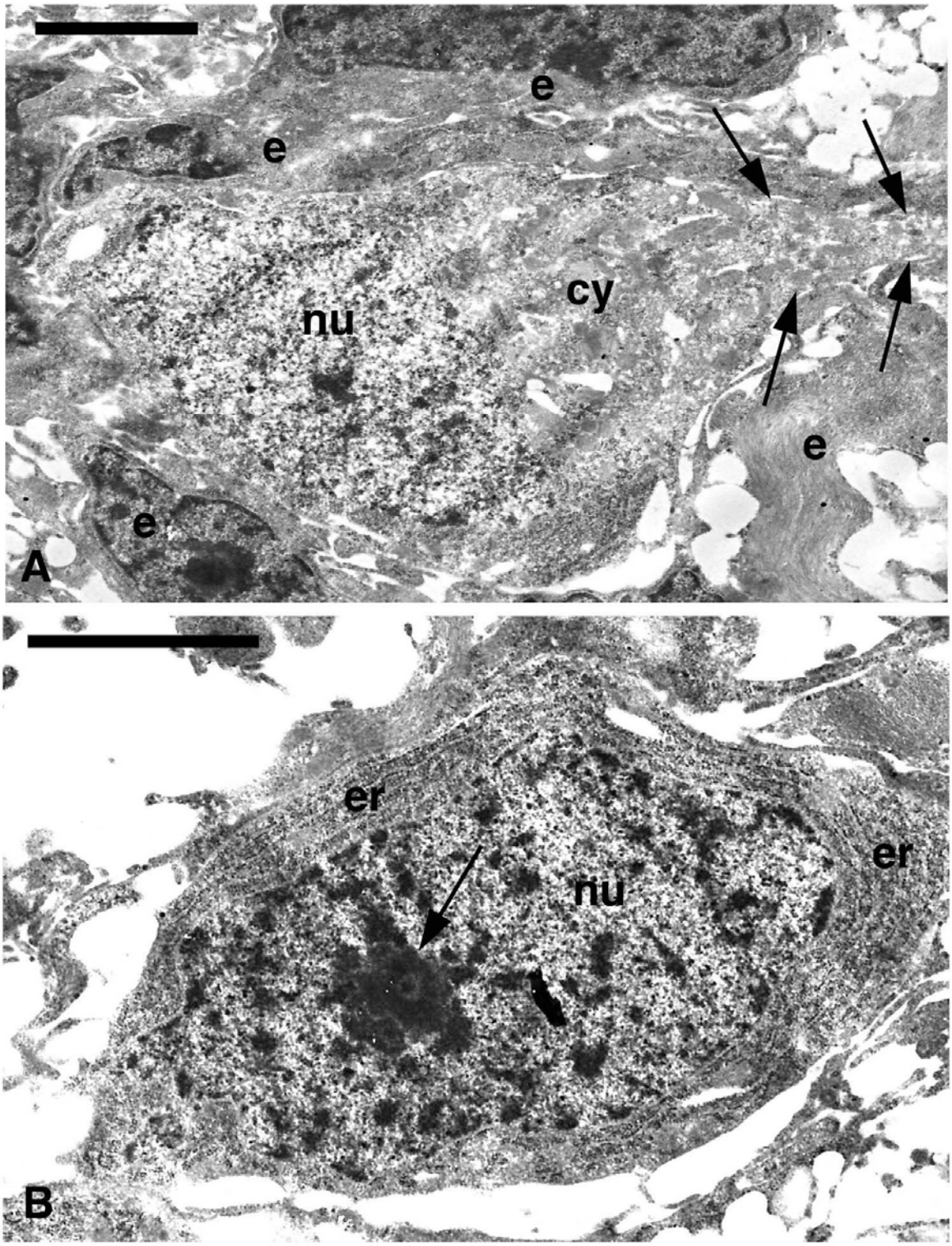

Figure 5. Two small neural cells located laterally to the ependyma (**A, B**). **A**, electron pale cell with a narrow elongation (arrows) still contacting ependymal cells. Bar, 2.5 μm. **B**, other pale cells with large nucleolous (arrow) and developed ergastoplasm cistaernae located outside but connected to the ependymal epithelium. Bar, 2.5 μm. **Legends**: cy, cytoplasm; e, ependyma; er, cisternae of the endoplasmic reticulum; nu, nucleus

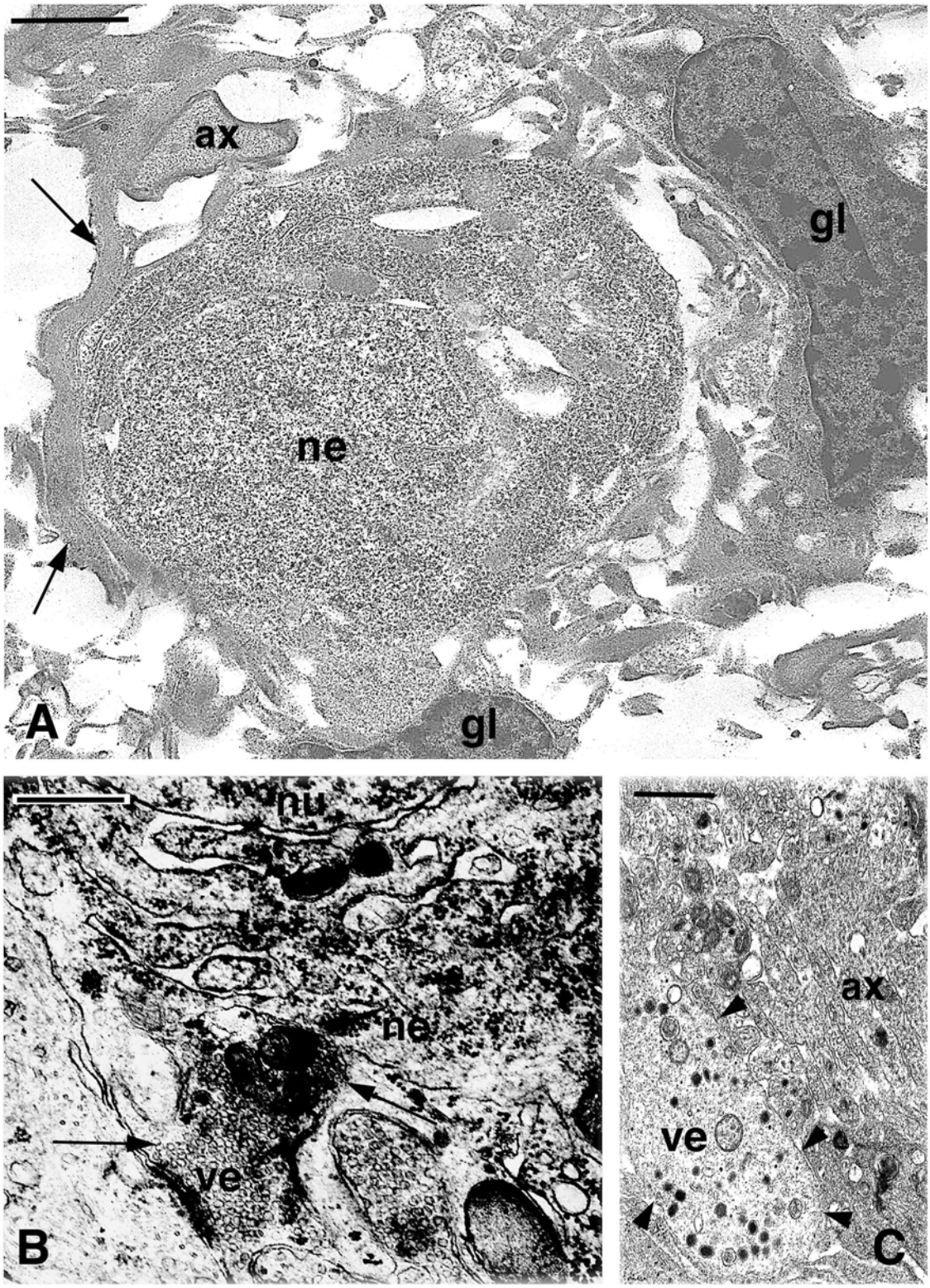

Figure 6. Nerve cells localized externally to the ependyma of rostral SC stump near the bridge at 29 days post-lesion. **A**, neuron with euchromatic nucleus and most free ribosomes but few cisternae of endoplamic reticulum. Myelinating axons are contacted by glial or ependymal elongations (arrows). Bar, 2 μm. **B**, detail of a synaptic bouton containing round vesicles contacting the cell body of a neuron (arrows). Bar, 0.5 μm. **C**, extra-cellular space between ependymal cells showing a group of thin axons containing microtubules close to a pre-terminal bouton containing dense core vesicles (arrowheads). Bar, 0.5 μm. **Legends**: ax, axons; gl, glial cell; ne, neuron; nu, nucleus; ve, synaptic vesicles

Other pale cells, previously observed with the light microscope (arrowheads in Figure 3 F, G), were instead identified as maturing or differentiated astrocytes. These astrocytes were electron-pale with an oval or more irregular nucleus showing one or more infoldings, and containing a variable amount of heterochromatin (Figure 7 A, B). Their cytoplasm contained mainly free ribosomes or sparse polysomes, but only occasional endoplasmic cisternae. The cytoplasm contained a variably developed cytoskeleton made of intermediate filaments, as it is characteristics for these cells (inset in Figure 7 A). No terminal boutons contacted these cells.

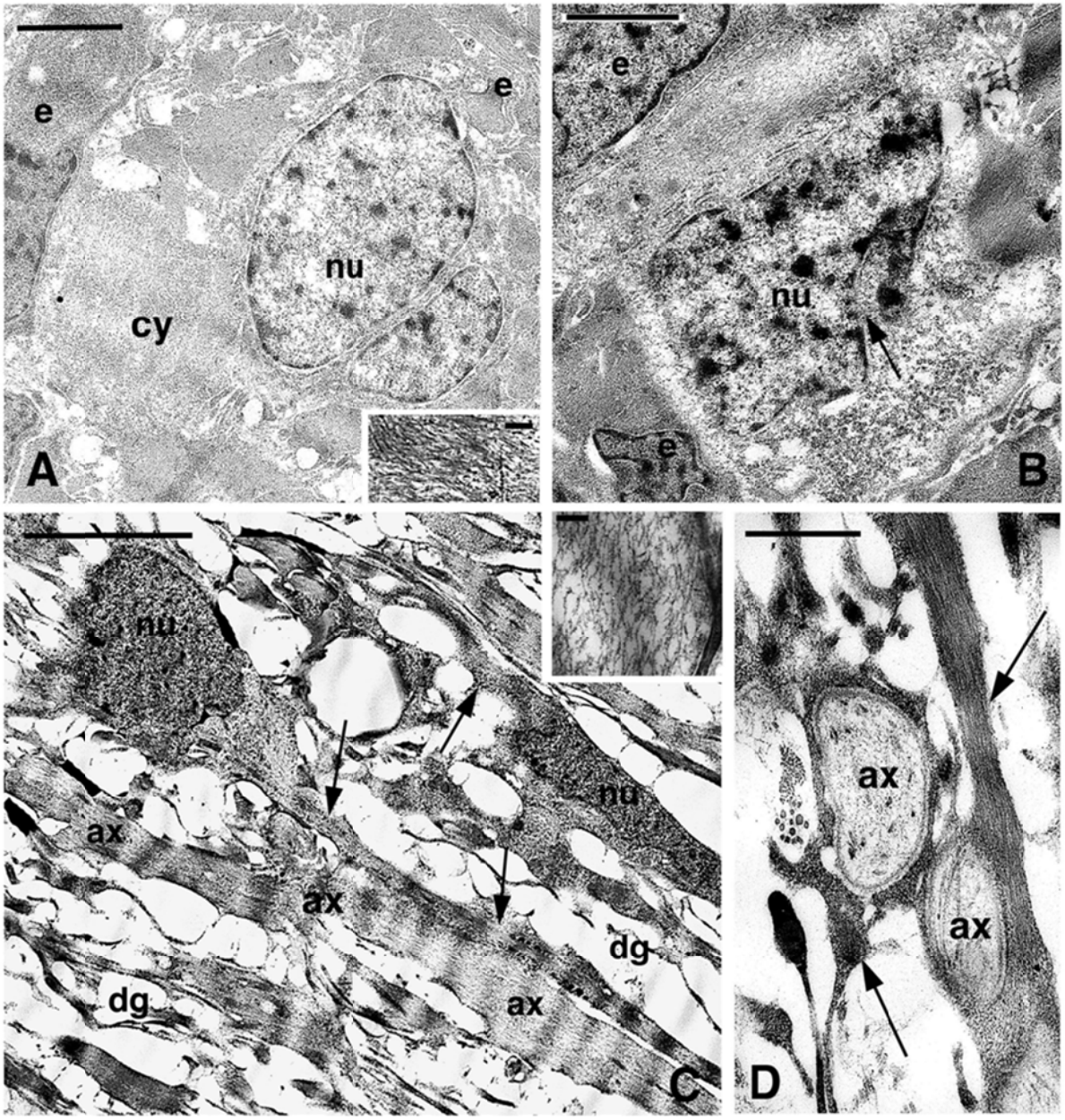

Figure 7. Astroglial cells among the stratified ependymal epithelium (**A, B**) or in the bridge area (**C, D**) at 29 days post-injury. **A**, pale cell rich in intermediate filaments among ependyma. (the inset details the intermediate filaments. Bar, 100 nm). The nucleus shows sparse small clumps of heterochromatin. Bar, 2 μm. **B**, Another pale cell localized among ependymal cells with folded (arrow) nucleus containing heterochromatine clumps. The cell contains numerous intermediate filaments, ribosomes and poly-ribosomes but very scarse cisternae of endoplasmic reticulum. Bar, 2 μm. **C**, elongated oligodendroglial cells producing intermediate-filament rich elongations (arrows) that parallel an axon (the inset shows the neurotubules; Bar, 200 nm). Most neuropile is empty. Bar, 5 μm. **D**, detail of cross-sectioned regenerating axons surrounded by glial elongations (arrows). Bar, 1 μm. **Legends**: ax, axons; cy, cytoplasm; dg, degenerated neuropile; e, ependymal cells; nu, nucleus

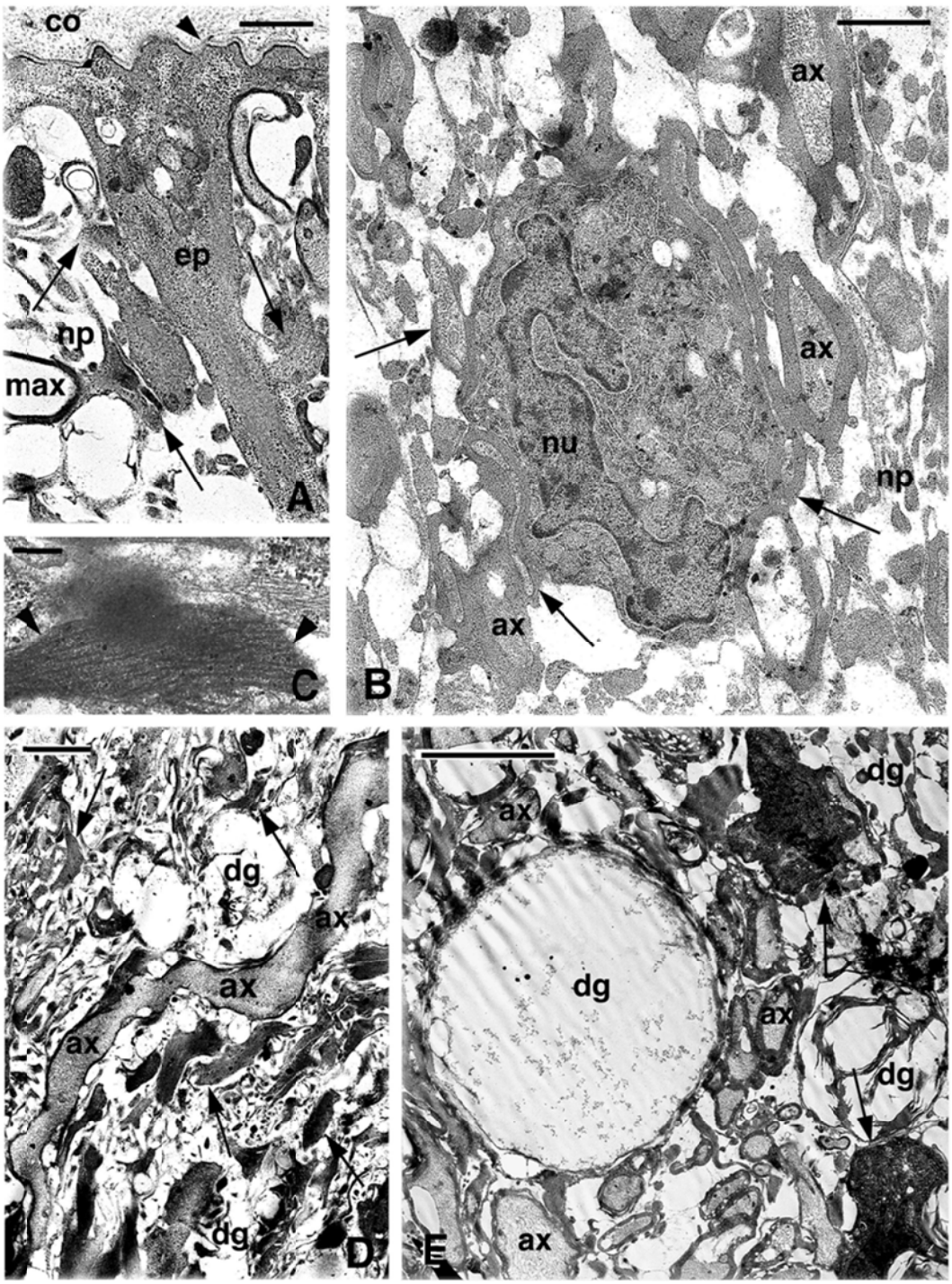

Figure 8. Myelinating cells in the peripheral bridge area between the SC stumps at 29 days post-lesion (**A-E**). **A**, terminal elongation (end-feet) of ependymal tanicytes onto the limiting basement membrane (arrowheads). Cytoplasmic elongations from tanycyte (arrows) surround the regenerated axons. Bar, 0.5 μm. **B**, electron-dense oligodendrocyte with irregular nucleus connected to regenerated axons (arrows). Bar, 1 μm. **C**, detail on dense oligodendrocyte arm (arrowheads) containing microtubules. Bar, 200 nm. **D**, regenerated axons partially myelinated, surrounded by degenerated axonal spaces mixed with glial elongations (arrows). Bar, 2 μm. **E**, other peripheral area of the bridge occupied by dense cells of the glia (arrows) close to myelinated axons. The largest vesicular areas represent mainly large or smaller degenerated axons. Also smaller myelinating axons (regenerating) are present. Bar, 5 μm. **Legends**: ax, axon; co, collagen fibrils; dg, degenerated (axonal/neuropilar) spaces; ep, peripheral process of ependymal tanicyte; max, myelinated axon; np, loose neuropile; nu, nucleus

In the white matter surrounding the ependymal ampulla, denser cells with oligodendrocyte characteristics were seen in both longitudinal and cross-section (Figures 7 C, D, 8). The neuropile around the latter small cells was very scarce, and numerous empty spaces were present among scattered darker cells and amyelinic or myelinated thin axons. The darker cells contained free ribosomes and microtubules and showed multiple contacting elongations with the surrounding axons (Figures 7 C, 8 A-C). Sparse myelinating axons within a scanty neuropile were observed up to the limiting external basement membrane where the peripheral end-feet of tanicytes terminated (Figures 7 D, 8 A). The nuclei of oligodendrocytes were often irregular, relatively dark and contained variably developed perinuclear chromatine. At 29 and 36 days post-lesion, most areas of the bridge tissue present underneath the external limiting membrane contained sparse cell debris and degenerating myelinated axons of small (0.4-1.0 µm) or larger dimension, confirming the light microscopic observations (2-4 µm, Figure 8 D). No large axons were seen in the bridge but instead the myelin sheath of large degenerated axons appeared still in the process of degradation after this long post-transection period (Figure 8 E). Close to degenerating cells and axons, small and very electron-dense cells, possibly representing microglia, were often present in these areas. Also in various areas of the bridge tissue, sparse axons of small caliber (0-5-1 µm large), some also in course of myelination and therefore likely regenerating, were seen (Figure 8 E). Therefore at 29-45 days after lesion ultrastructural indications of degenerating axons coexisting with regenerating axons were present in the bridge tissue.

4. Discussion

The present morphological and qualitative study shows that after 29-45 days post-lesion, the lumbar spinal cord in lizard recovers as a bridge composed by degenerated and sparse regenerating axons, among which oligodendrocytes and probably microglial cells are present. The scarcity of neuropilar elements (axons, dendrites, glial processes) indicates that the neuropile of the original spinal cord is partially regenerated in the bridge tissue where numerous figures of cells degeneration are still seen at 29-45 days post-lesion.

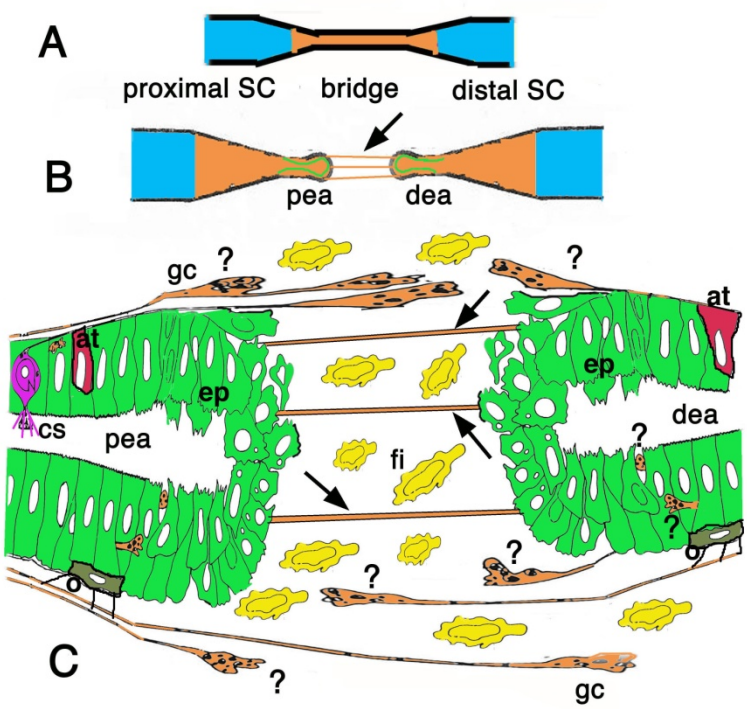

Figure 9. Schematic drawing summarizing the composition of the bridge region between two dilated ependymal ampullae of the SC stumps. **A**, longitudinal view of the bridge. **B**, detail of the proximal and distal ependymal ampullae with connecting axons (arrow). **C**, further cytological detail of the dilated terminal ampullae showing the presence of axons (arrows) among the connective tissue of the bridge. The regenerating axonal boutons passing the ampullae may be ascending or descending (?). **Legends**: at, astrocyte; cs, cerebro spinal fluid contacting neuron; dea, distal (caudal) ependymal ampulla; ep, ependyma; fi, fibroblast; gc, growing cone; o, oligodendrocyte; pea, proximal (rostral) ependymal ampulla

Sparse amyelinic axons and variably myelinated axons are present within the bridge indicating they are regenerating and may reconnect the two stumps of the spinal cord producecd after transection (Figure 9). The present study confirms previous histological and tract-tracing observations indicating that the two stumps remained after spinal cord transection become re-connected by sparse axons, allowing a limited recovery of hind limb movements (Raffaelli and Palladini, 1969; Alibardi, 2014). Previous studies on vertebrates with a good recovery after spinal cord transection (fish, newts and anuran larvae) have shown the regeneration of a limited number of axons crossing the transection gap (Stensaas, 1983; Bernstein, 1988; Davis et al., 1990). These axons, although less numerous than those of the original spinal cord, are however sufficient to recover the swimming or stepping movement after 2-4 weeks from paralysis. Recent studies have also indicated that turtles, following thoracic spinal cord transection, after about 20 days of limb paralysis can later recover some stepping movements (Reherman et al., 2009). In turtles, a bridge tissue is produced after 20-30 days post-lesion, showing similar ultrastructural images of axonal growth and myelination as here observed in the lizard (Reherman et al., 2011). Tract tracing studies have shown that the bridging axons originate from dorsal root ganglion neurons or/and inter neurons localized in the proximal stump of the spinal cord. The regeneration of bridge axons from inter neurons sparse in the grey matter of the proximal stump of the spinal cords has been also observed in lizard with lumbar transection (Alibardi, 2014). Inside the bridge, it is likely that further to oligodendrocytes, also some ependymal tanicytes can participate to the process of myelin formation, as radial glia or for transformation into oligodendrocytes, a process also advocated for the tail spinal cord of the ancient reptile tuatara (*Sphenodon punctatus*) and of lizards (Alibardi, 1990-91).

Previous studies on spinal cord lesions in the tail of lizards indicated that, in conjunction with the regeneration of new tail tissues, the new cord forms a simplified ependymal canal surrounded by some hundreds of descending axons, and by few glial and specialized cerebro-spinal fluid contacting neurons (Simpson, 1968; Egar et al., 1970; Alibardi and Sala, 1989; Alibardi. 1990-91, 1993; Simpson and Duffy, 1994). In the tail the innervation of the new tissues such as epidermis, dermis, blood vessels and especially of the large masses of regenerating muscles, from the regenerated spinal cord is impeded by the formation of a continuous cartilaginous tube which does not allow the exit of nerve roots (Bellairs and Bryant, 1985; Alibardi, 2010). The regenerated spinal cord remains simplified inside this cartilage while the motor and sensory innervation of the regenerating tissues derives from the last three segments and relative ganglia of the original cord (Terni, 1920; Cristino et al., 2000a,b). In the lumbar or thoracic cord, the absence of a permissive environment for innervation, namely the lack of surrounding regenerating tissues acting as targets for the sprouting of new nerves, was believed to limit the potential neurogenetic capability of the reptilian ependyma (Simpson, 1983; Simpson and Duffy, 1994). The present study, as well as recent data on the neurogenic potential of the spinal cord in turtles (Reherman et al., 2009, 2011), suggests that also ependymal cells of lumbar and thoracic spinal cord maintain the potential for neuroglial regeneration. Although small-medium size neurons within the rostral spinal cord stump appear to give rise to some of the crossing axons (Alibardi, 2014) it remains to be specifically analyzed whether they are connected to the local circuits of the locomotor pattern generator (Miller and Scott, 1977; Grillner, 1983; Bernstein, 1968).

In the present study the transected spinal cord was located at about 1-1.5 cm from the regenerating tail while in previous studies was present the normal tail (Furieri, 1957; Raffaelli and Palladini, 1969). In another lizard, *Anolis carolinensis* with intact tails, no limb recovery was reported after transection of the thoracic spinal cord (Simpson 1983). It is unknown whether the more intense cell regeneration observed in the present study derives from the vicinity with the regenerating tissues of the new tail to the transected area. This relative short distance between the lesion and the regenerating tiassues might offer a permissive environment for the regeneration and extensive growth of axons within the bridge between the two stumps of the interrupted spinal cord (Figure 9). Future molecular studies will address this problem.

In conclusion, together previous studies (Raffaelli and Palladini, 1969; Alibardi, 2014) the present ultrastructural study indicates that the neurogenic and gliogenic potential of the injured lumbar ependyma of lizards is similar to that of the tail, suggesting that likely stem ependymal cells (Alibardi, 1993, 2014; Zhou et al., 2012) or the dedifferentiation of ependymal cells after injury maintain the ability to repair the cord and form a bridge tissue containing connecting axons between the proximal and distal stumps. It remains to be studied how the surrounding or connected tissues, distal to the point of spinal cord transection may influence the growth of the few axons that cross the bridge and whether these axons connect to interneurons of the intrinsic locomotor generator system of the cord.

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