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REVIEW

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Cerebrospinal fluid-contacting neurons in the regenerating spinal cord of lizards and amphibians are likely mechanoreceptors

Lorenzo Alibardi

Comparative Histolab Padova, Department of Biology, University of Bologna, Bologna, Italy

Correspondence
Lorenzo Alibardi, Comparative Histolab Padova, Department of Biology, University of Bologna, via Semi 3, 40126 Bologna, Italy
Email: lorenzo.alibardi@unibo.it

Abstract

During spinal cord (SC) regeneration in the tail of amphibians and lizards, small neurons in contact with the central canal and cerebrospinal fluid (CSF) are formed. The present review summarizes previous and recent studies that have characterized most of these neurons as cerebrospinal fluid-contacting neurons (CSFCNs), especially in the regenerating caudal SC of lizards. CSFCNs form tufts of stereocilia immersed in the CSF, secrete exosomes, and are often in contact with a secreted protein-rod indicated as Reissner fiber. Ultrastructural, autoradiographic, immunohistochemical, and behavioral studies strongly indicate that most of these cells are mechanoreceptors that differentiate from ependymal cells within 20–30 days after SC amputation. Numerous CSFCNs are gamma amino-butyric acid (GABA)-ergic, uptake amino acids, receive few synaptic boutons, and contain neurofilaments, fibroblast growth factor (FGFs), and other signaling proteins, the latter likely secreted into the central canal. Similar neurons are formed in the SC of the tuatara (*Sphenodon puctatus*), anurans, and urodeles during tail regeneration. In lizard, most of their projection remains in the SC close to the regenerated tail, but they form synapses with neurons that receive descending nerves from the brainstem, including vestibular nuclei. CSFCNs, aside a possible neurosecretory activity, might sense liquor movements for maintenance of balance, a role that is supported from recent studies on other caudate vertebrates. The regeneration of these cells also in the nervous system of other vertebrates remains unknown.

KEYWORDS
cerebrospinal fluid-contacting neurons

1 | BACKGROUND ON SPINAL CORD REGENERATION IN LIZARDS

The tail of amphibians and reptilian sauropsids has multiple roles including swimming and balancing; these two activities require a coordination of neuromuscular circuits. The loss of the tail in urodeles, larval anurans, and in numerous lizards initially impairs the movements in these vertebrates, but this shortage is rapidly recovered through a process of complete or partial regeneration (Alibardi, 2010; Bellairs & Bryant, 1985; Carlson, 2007; Goss, 1969). While in amphibians numerous neurons and a broad anatomical restitution of the amputated spinal cord (SC) occur (Chernoff, 1996; Chernoff, Sato, Corn, & Karcavich, 2002; Ferretti, Zhang, & O'Neil, 2003; Filoni & Bosco, 1981; Tanaka & Ferretti, 2009), a simplified SC is formed during tail regeneration of lizards (Alibardi, 1990; Delorme & Vickaryous, 2017; Simpson & Duffy, 1994). In fact, the caudal SC entering the regenerating tail loses most of the gray and white matter and is reduced to a prevalent ependymal epithelium surrounded by few nerves and

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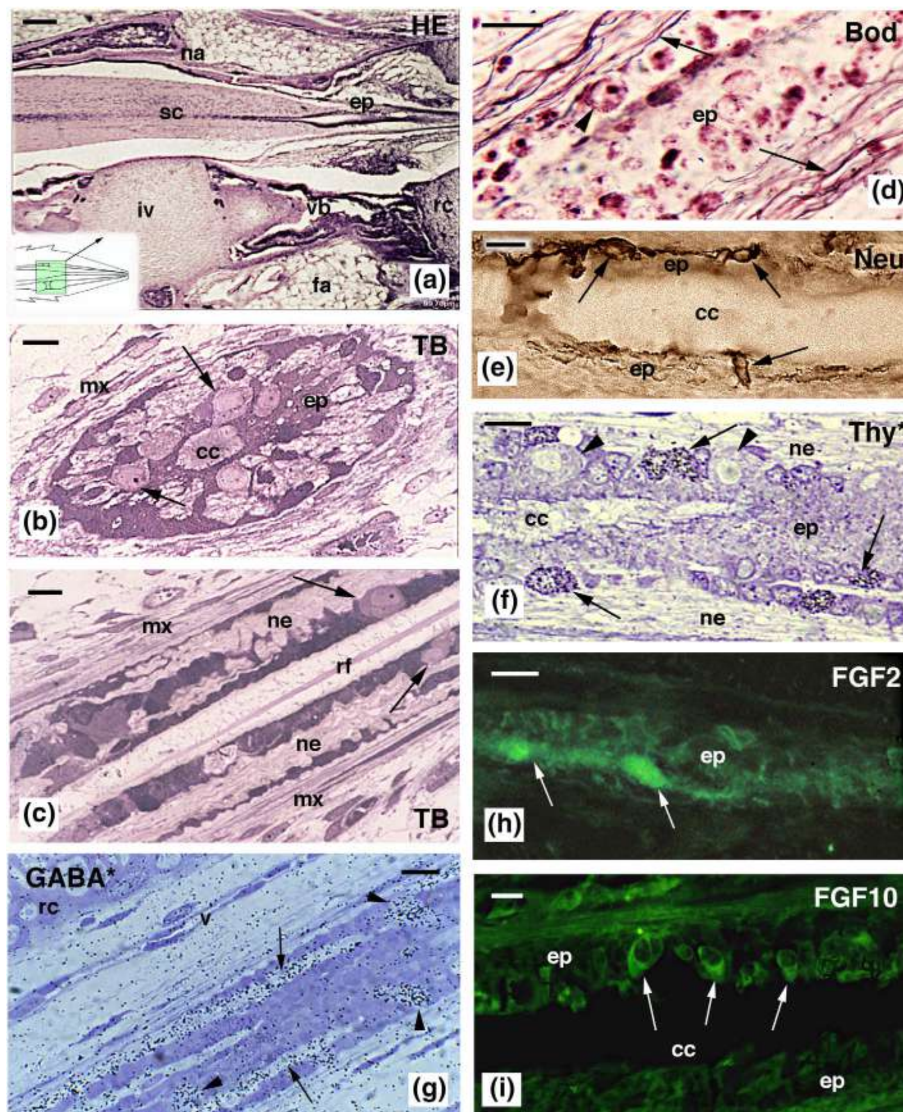

FIGURE 1 Microscopy images of regenerating caudal SC in lizards. (a)Passage area (see inset) between normal and regenerating SC in *Hoplodactylus maculatus*: Hematoxylin–eosin stain; bar, 100 μm. (b) Cross-section of ependyma in *Leiolopisma nigriplantare maccanni* with CSFCNs (arrows); Toluidine blue (TB) stain; bar, 10 μm. (c) Longitudinal section of ependyma in *L. nigriplantare maccanni* showing CSFCNs (arrows) contacting the Reissner fiber in the central canal; TB stain; bar, 10 μm. (d) Bodian stain of ependymal with peripheral nerves (arrows) in *Podarcis sicula* (the arrowhead points a neuron); bar, 10 μm. (e) Longitudinal section of ependymal in *Podarcis muralis* with cells immunolabeled (arrows) for neurofilaments (Neu, horse radish peroxidase-diamine benzidine (HRP-DAB) stain); bar, 10 μm. (f) Autoradiography of ependyma in *Lampropholis delicata* showing labeled ependymal cells (arrows) but not CSFCNs (arrowheads) 3–4 hr postinjection of tritiated thymidine (Thy*); bar, 10 μm. (g) Autoradiography of ependyma with labeled neurons (arrowheads) and nerves (arrows) in *Anolis carolinensis* 3 hr after injection of tritiated gamma-aminobutyric acid (GABA)*; bar, 10 μm. (h) Detail on two fluoresceine isothiocyanate (FITC)-immunofluorescent cells for fibroblast growth factor 2 (FGF2) (arrows) within the ependymal of *L. delicata*. Bar, 10 μm. (i) fibroblast growth factor 10 (FGF10)-immunolabeled CSFCNs (arrows) in the ependyma of *P. muralis*; bar, 10 μm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neurons; ep, regenerating ependyma; fa, fat tissue; iv, intervertebral cartilage; mx, meninx; na, neural arch; ne, nerves (groups of regenerating axons) present among ependymal cells; rc, regenerated cartilage; rf, Reissner fiber; SC, normal spinal cord; vb, vertebral body

encased by an almost continuous cartilaginous tube (Figure 1a–d). The latter isolates the regenerated SC from the surrounding tissues formed in the new tail that are instead innervated from neurons located in the three neuromeres of the SC and spinal ganglia of the original tail that are closer to the regenerating tail (Cristino, Pica, Della Corte, & Bentivoglio, 2000a; Cristino, Pica, Della Corte, & Bentivoglio, 2000b; Geuna, Giacobini-Robecchi, Poncino, & Filogamo, 1992).

Most studies before 1980s indicated lack of neural regeneration in the caudal SC of lizards, whereas only ependyma and few glial cells were formed (Egar, Simpson, & Singer, 1970; Simpson, 1968). However, following ultrastructural, autoradiographic, and immunohistochemical studies showed the presence of few small neurons, 12–15 μm large, among the prevalent ependymal population, and characterized most of these cells as cerebrospinal fluid-contacting

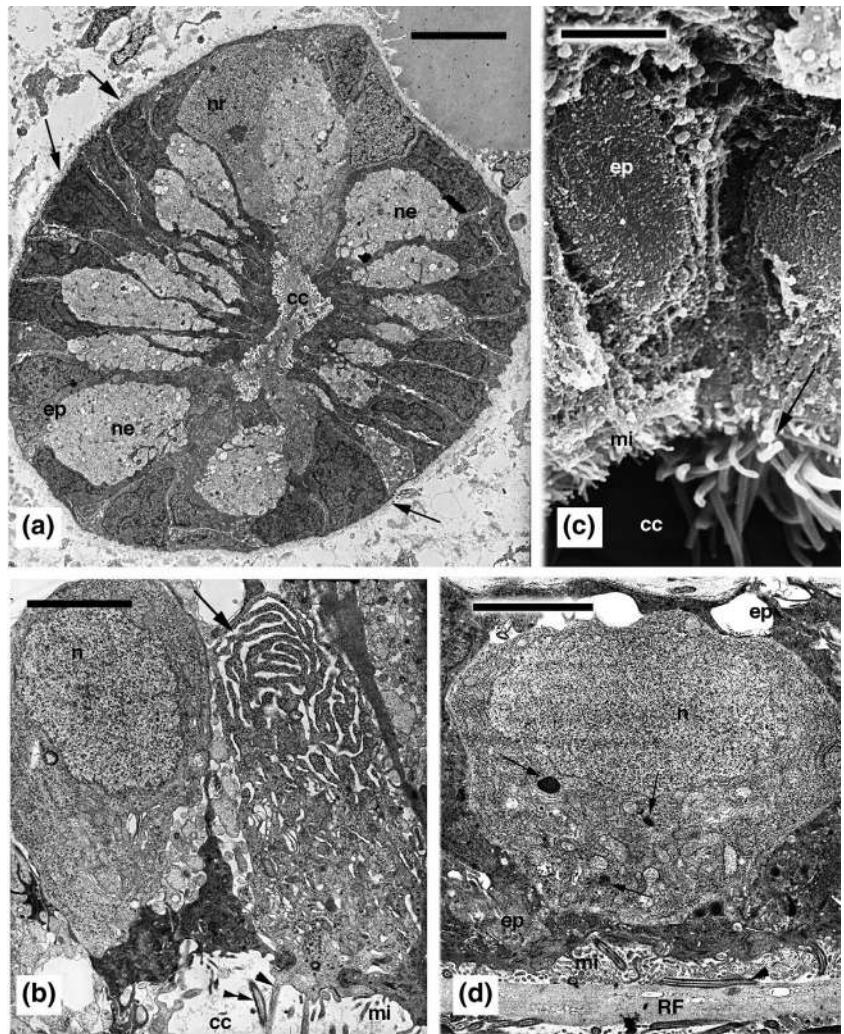

FIGURE 2 Transmission (a,b,d) and scanning (c) electron microscopy of regenerated SC with CSFCNs. (a) Cross-view of regenerated ependymal tube in *Leiolopisma nigriplantare maccanni* at 2 months of tail regeneration. Arrows point to the external basement membrane; bar, 10 μm. (b) Details on two electron-pale CSFCNs among denser ependymal cells in *L. nigriplantare maccanni.* The arrow indicates a cytoplasmic region rich in ergastoplasm. The arrowhead point stereocilia while the double arrowhead indicates a cilium; bar, 5 μm. (c) Detail showing tufts of stereocilia (arrow) in the ependymal lumen of *Anolis carolinensis* at 2 months of tail regeneration; bar, 5 μm. (d) CSFCN in *Lampropholis delicata* at 2 months of regeneration storing dense granules (arrows) and close to the Reissner fiber that is contacted by numerous cilia (arrowhead); bar, 2.5 μm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neurons; ep, ependymal cell/cytoplasm; mi, microvilli; n, nucleus; ne, nerves (groups of regenerating axons) present among ependymal cells; nr, neuron/neuronal cytoplasm (CSFCN); RF, Reissner fiber; SC, normal spinal cord

neurons (CSFCNs; Alibardi & Meyer-Rochow, 1988; Alibardi & Sala, 1986, 1988; Alibardi, Wibel, & Simpson, 1993; Simpson & Duffy, 1994; Figures 1e–i and 2). CSFCNs were broadly known in the normal SC and cerebral ventricles of all vertebrates (Vigh & Vigh-Teichman, 1973). In particular, in rats and mice recent studies have shown that numerous neurons sending their dendritic endings into the cerebrospinal fluid (CSF) have their cell body located in the ventral gray matter of the fourth ventricle and in the mesencephalic ventricle (Silvio aqueduct), forming a nuclear structure indicated as CSF-contacting nucleus (Song & Zhang, 2018). While the formation of CSFCNs during embryogenesis is known for some vertebrates (summarized in Petracca et al., 2016), no information was available on the regeneration of these cells under physiological or traumatic conditions.

Comparative ultrastructural studies showed that these cells are present in the regenerating caudal SC of lizards belonging to the main families, including *Podarcis muralis*, *Podarcis sicula* and *Lacerta viridis* (lacertids), *Tarentula mauritanica*, *Hemidactylus maculatus*, *Lygodactylus capensis*, *Sphaerodactylus argus*, *Eublepharis macularious* (geckonids), *Leiolopisma nigriplantare maccanni*, *Scincella lateralis*, *Lampropholis delicata* and *Lampropholis guchenoti* (scincids), and *Anolis carolinensis* and *Anolis lineatopus* (iguanids). Also the ancient lizard-like tuatara, *Sphenodon punctatus*, differentiates these cells in the SC during tail regeneration (Alibardi & Meyer-Rochow, 1990). Finally some urodele amphibians (*Triturus vulgaris*, *Triturus cristatus*, and *Pleurodeles waltl*), and in anurans (*Rana dalmatina* and *Xenopus laevis*) aside from motor and sensory neurons of the gray matter, also CSFCNs are regenerated (Alibardi, 1989, 1990, 1990–1991). These comparative studies, summarized in the present review indicated that CSFCNs have a broad capability of regeneration in tailed vertebrates and that they are likely mechanoreceptors. The role of these neurons in the physiology of movements and pH control in caudate vertebrates has been recently addressed (Bohm et al., 2016; Fidelin et al., 2015; Jalalvard, Robertson, Wallen, & Grillner, 2016; Jalalvard, Robertson, Wallen, Hill, & Grillner, 2014; Orts-Del'Immagine & Wyart, 2017; Wyart et al., 2009).

2 | CYTOLOGICAL CHARACTERISTICS OF CSFCNS

The regenerated CSFCNs show the typical aspects described for those present in brain ventricles especially of the hypothalamus and

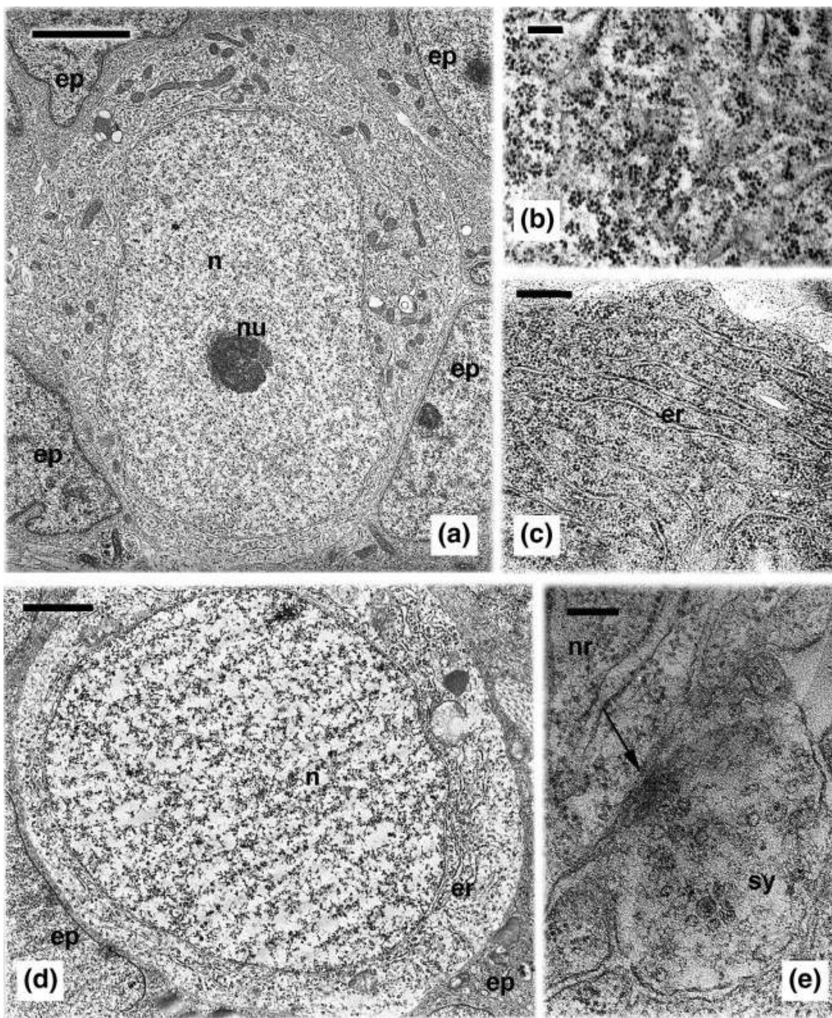

FIGURE 3 Transmission electron microscopy views of differentiating CSFCNs within the ependymal tube at 1–2 months of tail regeneration. (a) General view of and electron-pale early differentiating neuron in *Hoplodactylus maculatus*; bar, 2 μm. (b) Higher magnification showing most polyribosome in neuroblast of *Podarcis sicula*; bar, 200 nm. (c) Developing cisternae of rough endoplasmic reticulum in CSFCN of *Leiolopisma nigriplantare maccanni*; bar, 0.5 μm. (d) Developing perinuclear ergastoplasm in pale neural cell of *P. sicula*; bar, 1 μm. (e) Synaptic bouton with pleomorphic vesicles apposed (arrow) to a CSFCN in *Lampropholis delicata* at 2 months regeneration; bar, 200 nm. CSFCNs, cerebrospinal fluid-contacting neurons; ep, ependymal cell; er, ergastoplasm/rough endoplasmic reticulum; n, nucleus; nr, neuron/neuronal cytoplasm; nu, nucleolus; sy, synaptic Bouton

in the SC (Vigh & Vigh-Teichman, 1973). The regenerating cord consists initially in a pseudo-stratified layer of columnar ependymal cells that become ependymal tanycytes after 2–3 months of regeneration. Tanycytes feature a luminal cytoplasm facing the central canal of the SC and a narrow cytoplasmic and peripheral elongation that accumulates bundles of intermediate filaments and terminates against the basement membrane surrounding the SC (Alibardi, 1990; Delorme & Vickaryous, 2017; Simpson, 1968; Turner & Singer, 1973; Figures 1 and 2). Outside the basement membrane is present a thin meninx that often contains long elastic fibers in addition to collagen fibrils, important elements for contrasting the bending of the narrow SC (Alibardi & Meyer-Rochow, 1988, 1990; Egar et al., 1970; Simpson, 1968; Figure 2a). Some hundreds of axons, including local sprouting, and ependymoglial elongations are localized between the ependymal cells and, later, spaces formed between tanycytes, true gutters along which these axons can grow distally during their regeneration (Simpson, 1970, 1983; Simpson & Duffy, 1994).

The cytology of regenerated CSFCNs has been extensively analyzed, from early stages as neuroblasts to fully mature neurons in numerous lizard species (Alibardi, 1990, 1993; Alibardi & Meyer-Rochow, 1988; Alibardi & Sala, 1986, 1988; Alibardi, Wibel, et al., 1993; Figures 2 and 3). Briefly, during their differentiation from ependymal cells, neural cells become electron-pale, their nucleus turns rounded and euchromatic with a large nucleolus, their size increases in comparison to ependymal cells and the cell tends to assume an oval to pear-like shape (Figure 3a). Initially, the cytoplasm contains only polyribosomes but rapidly sparse endoplasmic cisternae are formed and occupy part of the cytoplasm, especially close to the nucleus as a typical secretory cell (Figures 2b,d and 3b–d). The Golgi region, present near the nucleus and facing the luminal side of CSFCNs, shows some stacks of dictyosomes that produce numerous pale vesicles, dark granules of glycoproteic secretory material, and 90–150 nm large dense core-vesicles of peptidergic type. Also multivesicular bodies, some possibly representing exosomes before discharge into the extracellular space, are commonly observed in the luminal cytoplasm of CSFCNs, and in their surface communicating with the central canal. Occasional axosomatic synaptic boutons containing round or pleomorphic/flat vesicles are seen on the nonluminal cell body, forming mainly symmetric (inhibitory) but also asymmetric (excitatory) synapses (Figure 3e). The apical cytoplasm typically contains a tuft of stereocilia and one or two cilia, often in contact with the Reissner fiber,

a glycoproteic secreted rod present in the central canal of the SC (Alibardi & Meyer-Rochow, 1988; Alibardi, Wibel, et al., 1993; Figure 2c). The presence of stereocilia connected to a cytoskeletal meshwork and located in the luminal cytoplasm of CSFCNs are typical characteristics for mechanoreceptors.

3 | CSFCNs UPTAKE AMINO ACIDS AND ARE GABA-ERGIC

The neural nature of these cells, representing a low percentage in different species (3–10%) of the cells forming the regenerated SC in lizards, was further demonstrated through daily injections of GABA for about 3 weeks to lizards during tail regeneration (Alibardi, Sala, & Meneghini, 1987; Figure 4). In the lizard *P. sicula* treated with GABA during tail regeneration the number of CSFCNs increased in comparison with controls, from 5.2 to 18.9% in *P. sicula* after 23 days of treatment (Alibardi et al., 1987; Figure 4a,b). Also their size, cytoplasmic differentiation, and the formation of synaptic boutons were increased after this treatment. The increment of secretory activity evidenced using light microscopy as glycoproteic material in the ependymal lumen (periodic acid reaction of shiff (PAS) positive) and was confirmed using electron microscopy (Ag–metenamine positive). In fact, the ergastoplasm was more developed in these larger CSFCNs, contained more numerous and longer cisternae of endoplasmic reticulum, a higher number of vesicles and granules from the larger Golgi apparatus. Also, a higher secretion of amorphous material and of multivesicular bodies (now interpreted as exosomes) was seen in the lumen (Figure 4c–f). The presence of synaptic boutons containing pleomorphic flat or round vesicles were more commonly detected on the cell body of CSFCNs in GABA-treated with respect to untreated lizards (Figure 4g).

The above observations suggested that one of the neurotransmitters present in these cells was GABA and/or other amino acids involved in the biosynthesis and catabolism of this neurotransmitter (Johnson & Roberts, 1984). This was verified after injecting tritiated GABA and/or proline into lizards during tail regeneration, two amino acids that can be metabolically converted into glutamate (Alibardi, 1995; Alibardi, Gibbons, & Simpson, 1993; Figures 5 and 6). The

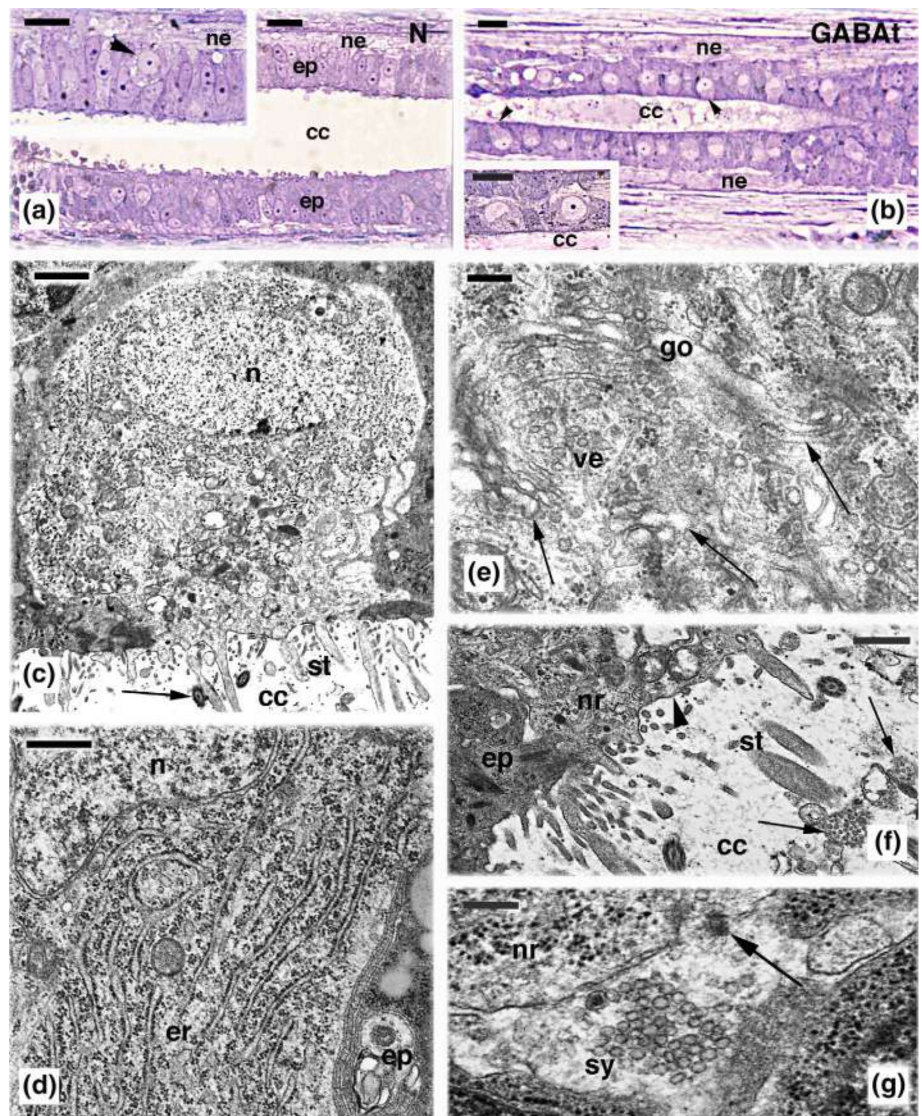

FIGURE 4 Light microscopy (a,b) and transmission electron microscopy images (c–g) of regenerating ependyma in normal (a) and after GABA treatment (b–g) for 23 days in *Podarcis* (*Lacerta*) *sicula*. (a) Normal ependymal (untreated, N) showing large prevalence of ependymal cells (in the inset; bar, 10 μm) only one neuron is indicated by an arrowhead). (b) Treated lizard showing numerous CSFCNs (arrowheads) and secreted material inside the central canal. The inset (bar, 10 μm) shows two large neurons rich in cytoplasmic granulations. (c) Low magnification view of secretory CSFCN with cilia (arrow) and stereocilia in the central canal; bar, 1 μm. (d) Detail showing a developed perinuclear ergastoplasm; bar, 0.5 μm. (e) Hypertrophic Golgi with numerous forming secretory vesicles (arrows) and numerous pale and coated vesicles; bar, 200 nm. (f) Luminal surface showing secretion of multivesicular bodies (arrowhead) and presence of exosomes (arrows) inside the central canal; bar, 0.5 μm. (g) Axosomatic bouton containing round and pleomorphic vesicles in contact (arrow) with a neuron; bar, 200 nm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neurons; ep, ependyma; er, ergastoplasm; GABAt, GABA treatment; go, Golgi apparatus; n, nucleus; ne, nerves (groups of regenerating axons) present among ependymal cells; nr, neuron; st, stereocilia; ve, vesicles; sy, synaptic bouton

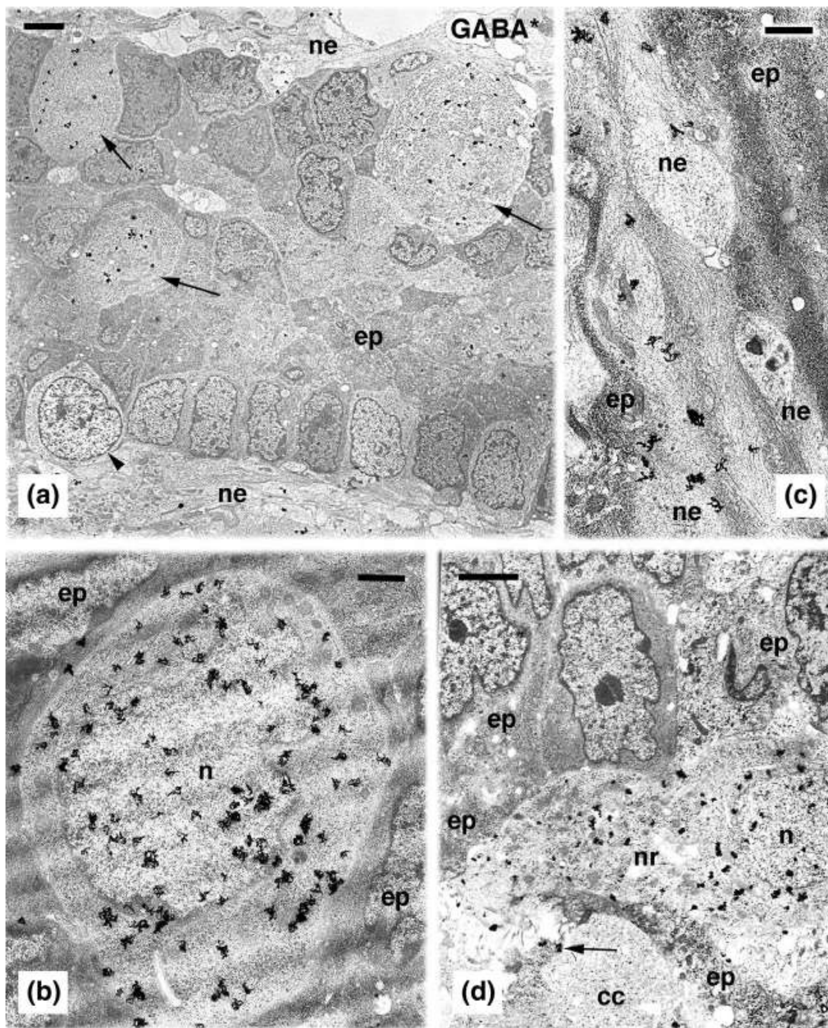

FIGURE 5 Transmission electron microscopic autoradiography of regenerating ependyma 3 hr after injection of tritiated GABA (GABA*) in *Scincella lateralis* (a) and *Anolis carolinensis* (b–d). (a) Three labeled neurons (arrows) and an unlabeled glial cell (arrowhead) are seen among unlabeled ependymal cells; bar, 2 μm. (b) Detail on a highly labeled neuron, including the nucleus; bar, 1 μm. (c) Higher magnification detail on labeled thin nerves (axons) located among ependymal cells; bar, 200 nm. (d) Intensely labeled, electron-pale CSFCN among unlabeled ependymal cells. The arrow points to trace labeling seen inside the central canal; bar, 2 μm. CSFCNs, cerebrospinal fluid-contacting neurons; ep, ependyma; n, nucleus; ne, nerves (regenerating axons); nr, neuron

results suggested that this neurotransmitter and also other amino acids could be utilized for studying the differentiation of these cells, and confirmed their neural nature. The ultrastructural autoradiographic study of the regenerating SC after injections of tritiated GABA in *A. carolinensis* and *S. lateralis* showed a specific and high uptake in CSFCNs, in sparse axons and in axosomatic synaptic boutons present among the ependymal cells, that instead remained unlabeled (Alibardi, Gibbons, et al., 1993; Figures 1g, 5, and 6a). Other pale cells with more heterochromatic nucleus and irregular shape, likely representing glial cells, remained instead unlabeled (Figure 5a). Using tritiated proline, another amino acid related to GABA metabolism (Johnson & Roberts, 1984) that was injected in the lizard *L. delicata* during tail regeneration, CSFCNs also appeared more intensely labeled than the other cells of the ependymal tube, indicating that CSFCNs are metabolically more active than ependymal cells (Alibardi, 1995; Figure 6b,c). The presence of labeling in the luminal cytoplasm, and sometimes also of few tracing autoradiographic signals within the central canal at 1 and 3 hr postinjection (Figures 5d and 6b,c), further suggested that CSFCNs secrete material into the CSF. The presence of GABA-ergic synapses, although of unknown origin, also confirmed the presence of inhibitory signals reaching CSFCNs (see below).

4 | CSFCNs DERIVE FROM EPENDYMAL CELLS

The origin of CSFCNs in the regenerating SC may be from local ependymal cells or, possibly, through the migration of neural cells from the original SC within the central canal of the regenerating SC. The differentiation of CSFCNs and their origin were analyzed using autoradiography and ependymal/SC autotransplants, both indicated a derivation from the regenerating ependyma.

Detailed ultrastructural autographic studies after tritiated thymidine injections showed that in *A. carolinensis*, *S. lateralis* and *L. delicata*, CSFCNs were unlabeled at 3–4 hr from the injection of the radioactive DNA nucleotide while several ependymal cells took up this precursor of DNA synthesis, indicating cells preparing for multiplication (Alibardi, Gibbons, et al., 1992; Figures 1f and 7a,b). The nuclei of some electron-pale cells with the characteristics of differentiating CSFCNs, as described before, appeared variably labeled at 12, 20, and 30 days postinjection, indicating labeling dilution due to cell division (Figure 7b), from the initially labeled ependymal cell precursors to the fully mature CSFCNs (Alibardi, 2014; Figure 7). From these studies, a period of 20–30 days for the complete differentiation of CSFCNs was

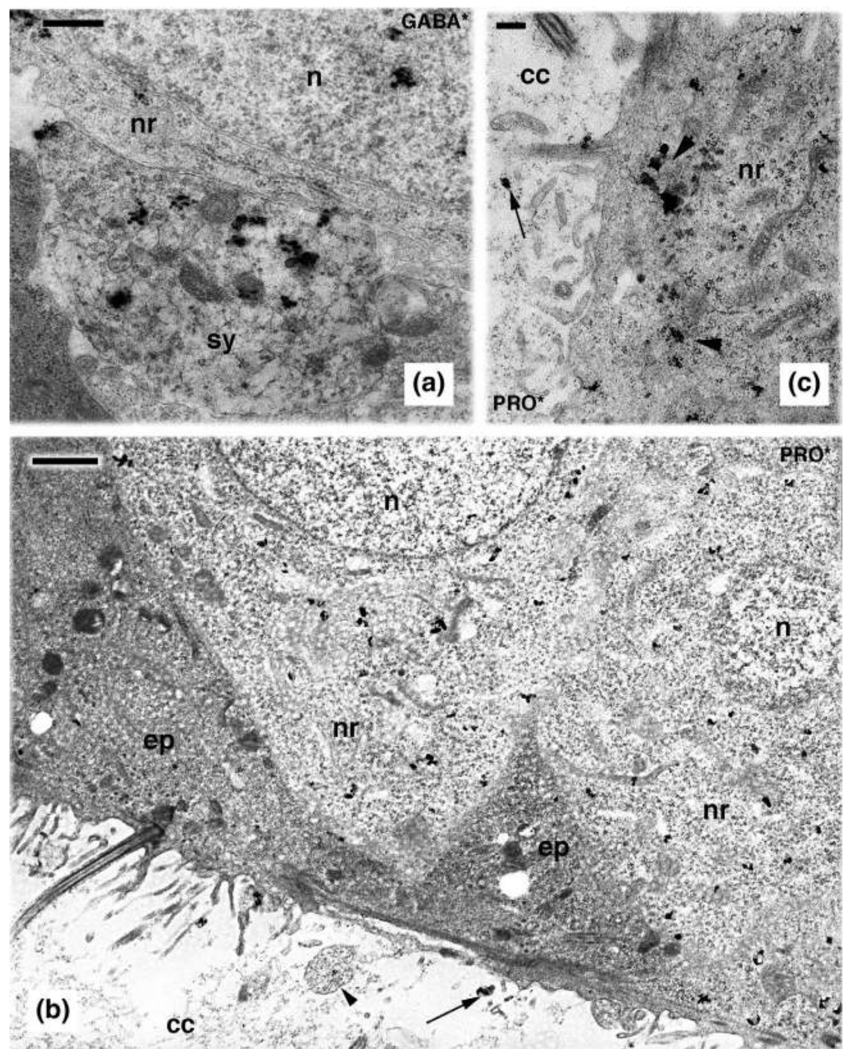

FIGURE 6 Transmission electron microscopy autoradiography for tritiated GABA (GABA*) in *Anolis carolinensis* (a) and proline (PRO*) in *Lampropholis delicata* (b,c) 3 hr after injection of the radioactive amnino acids. (a) Labeled axosomatic synaptic bouton mainly containing sparse polymorphic-flat vesicles onto a GABA-labeled neuron; bar, 0.5 μm. (b) Two paler labeled CSFCNs among ependymal cells. An exosome is seen in the central canal (arrowhead) where also trace grains are present (arrow); bar, 1 μm. (c) Detail of the luminal surface of a CSFCN with trace labeling (arrowheads), also seen in the lumen (arrow); bar, 250 nm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neurons; ep, ependyma; n, nucleus; nr, neural cell; sy, synaptic bouton

estimated, including synaptogenesis and formation of the stereocilia projecting into the ependymal lumen.

The origin of CSFCNs from ependymal cells was also tested by an indirect experiment that consisted in the autotransplant of the SC removed from the distal part of a normal tail or of the cartilage-ependyma removed from the regenerated tail (the cartilage-ependyma). The SC or cartilage-ependyma explants were immediately implanted in the tail stump of the same lizard (Alibardi, Sala, & Miolo, 1988; Figures 8 and 9d). In numerous cases one or two additional and smaller tails were formed (Figures 8a), and their histology and ultrastructure was analyzed at about 1 and 3 months after the operation. The implanted SC underwent a massive degeneration in 1–2 weeks and essentially only the ependymal tube remained (Figure 8b). In the implanted cartilage-ependyma, only ependymal cells were present in the smaller supernumerary tails so that any possible neurons found at 1 month from the implant could be only derived from these epithelial cells. The narrow ependymal tube present in the supernumerary tails consisted in a pseudostratified layer of ependymal cells that appeared as typical tanycytes at 1–3 months of regeneration, and few pale cells contacting the lumen were seen at 25 days (Figure 8b–f). Although not quantified, also few electron-pale axons containing parallel-oriented microtubules, much less numerous in the normal regenerated SC were seen among ependymal cells in the ependymal tubes of the supernumerary tails (Alibardi et al., 1988). Because only ependymal cells were present in the implants and no connection was formed with the normal SC, the few CSFCNs observed could only be derived from the implanted ependymal cells and not migrated from the SC of the tail stump. The spaces present among the ependymal elongations were largely empty or contained cell debris, although sparse and nonmyelinated axons mixed to glial-ependymal protrusions were also seen at 1 month postoperation (Figure 8c–e). At 3 months from the operation only ependymal tanycytes rich in bundles of intermediate filaments were present, suggesting that the pale neurons had largely degenerated. This result could be expected since the ependymal tube was completely isolated from central connections and peripheral tissues. The origin of CSFCNs from special groups of ependymal cells during early (fish, amphibians) or late (chick, rodents) stages of development has been shown in the SC, although their possible regeneration in adult vertebrates is not known (Petracca et al., 2016).

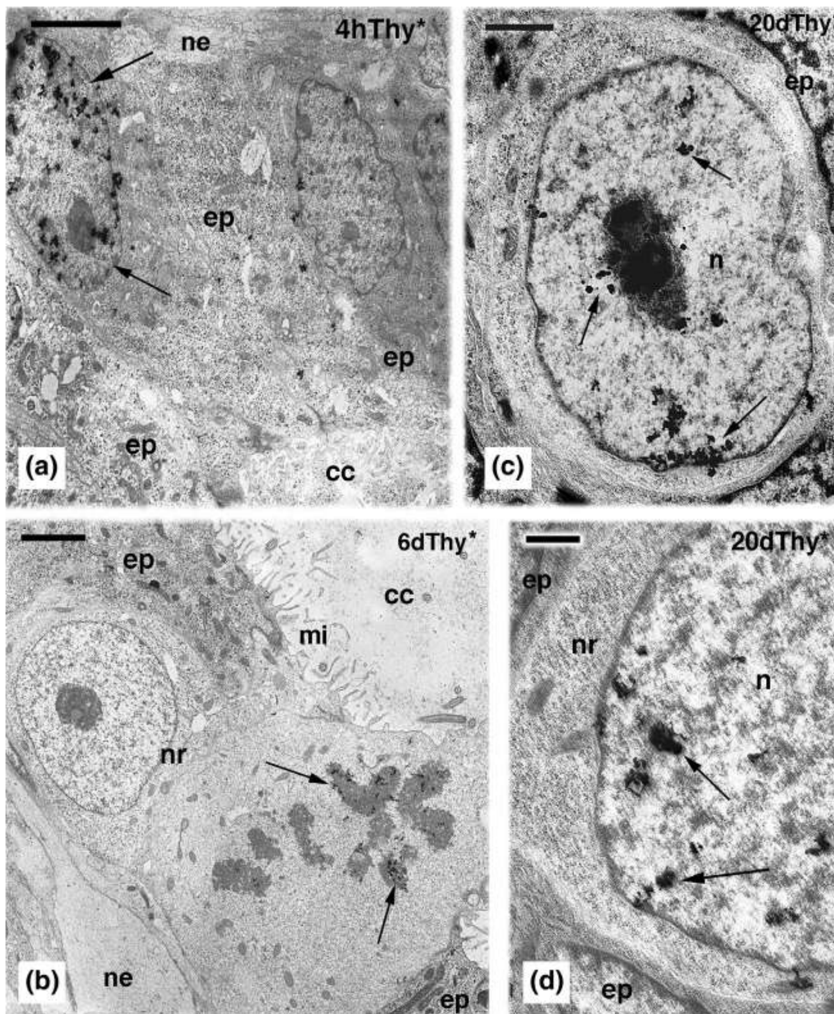

FIGURE 7 Transmission electron microcopy autoradiography of the regenerating ependyma in *Lampropholis delicata* after successive periods from the injection of radioactive thymidine (Thy*). (a) At 4 hr only ependymal cells show labeled nuclei (arrows); bar, 2 μm. (b) At 6 days postinjection only ependymal cells and dividing cells are labeled (arrows on labeled chromosomes, indicating labeling dilution); bar, 2 μm. (c) Young, still differentiating CSFCN 20 days postinjection showing some nuclear labeling (arrows); bar, 1 μm. (d) Detail on labeled nucleus (arrows) in electron-pale neuron 20 days postinjection; bar, 0.5 μm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neuron; ep, ependyma; mi, microvilli; n, nucleus; ne, nerves (axon bundles); nr, neuron

5 | IMMUNOHISTOCHEMICAL CHARACTERISTICS OF CSFCNs

Recent immunohistochemical studies for neuronal markers and neurofilaments in the regenerating caudal SC of lizards (geckos) further revealed a neurogenic potential for the caudal ependyma of these reptiles (Delorme & Vickaryous, 2017; Zhou et al., 2013; Figure 1d,e). In the Japanese gecko (*Gekko japonicus*) neuronal cells immunopositive for neuron-specific enolase appear around 3 weeks of regeneration (Zhou et al., 2013), a period corresponding to those determined by the above autoradiographic studies for CSFCNs. The labeling of some ependymal cells for the neuronal stem cell marker nestin suggests that neuronal stem cells are present in the normal and regenerating SC of lizards.

Additional strong support of the presence of neurogenic cell precursors in the SC of lizards derives from the immunohistochemical detection of the neural protein marker HuC/D in the regenerating tail SC of the leopard gecko *E. macularious* (Delorme & Vickaryous, 2017). In particular these authors found numerous CSFCNs, cells that they named as "Cerebrospinal fluid-contacting cells," at 18–30 days up to 140 days of tail regeneration, in addition to glial cells immunopositive for glial acidic fibrillar protein (GAFP). The latter study detected a heterogenous population of ependymal cells, among which some 5BrdU-positive slow cycling cells that are also immunolabeled for the neural stem cell marker SOX2. This study further indicated that among ependymal cells of lizards some subtypes are neural stem/progenitor cells (see Delorme & Vickaryous, 2017).

Other observations have also indicated that CSFCNs are immunolabeled for FGF-1, -2 and -10, for GABA, and that they uptake neuronal tracers such as horseradish peroxydase (HRP) and Lucifer yellow (Alibardi, 2016; Alibardi & Lovicu, 2010; Duffy, Hawrych, Liebich, & Simpson, 1993; Figures 1h–i and 9a,b). The common presence of multivesicular bodies that can now be interpreted as precursor of the exosomes detected in the central canal is particularly interesting for the role of the regenerating SC in the process of tail regeneration of lizards. In fact, exosomes can transport signaling proteins like Shh and Wnt that influence numerous developmental processes (McGough & Vincent, 2016). These observations may suggest that these secretory cells produce signaling proteins and exosomes for other functions aside from the likely role as mechanoreceptors. In fact, it has been hypothesized that the ependyma in the regenerating SC of the tail in lizards produces a neurotrophic signal that stimulates the growth of the regenerative blastema and in particular the induction of the cartilaginous tube (Alibardi et al., 1988; Lozito & Tuan, 2016; Simpson,

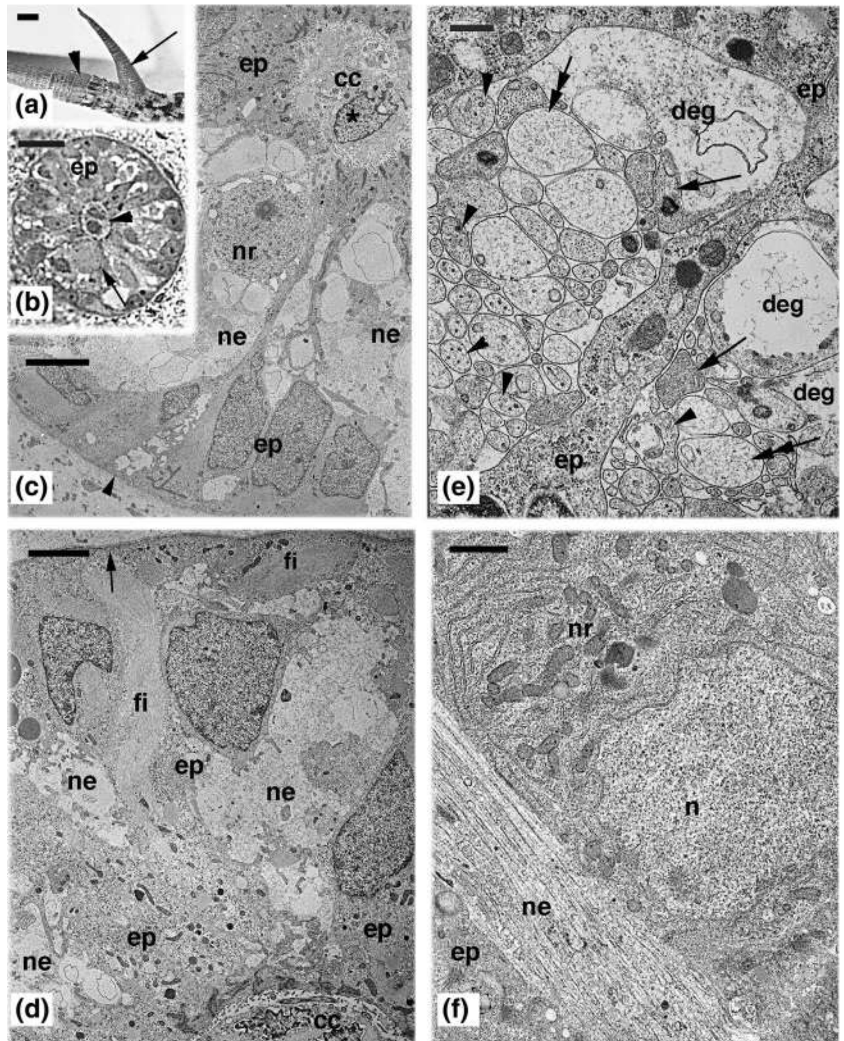

FIGURE 8 Cell composition of the regenerated ependymal inside supernumerary tails (a) in *Podarcis muralis*, with relative cross-sectioned histology (b) and electron microscopy (c–f) at 1 month postimplant. (a) After 1 month from the autotransplant of cartilage-ependyma, a supernumerary (extra) tail is formed (arrow) in addition to the normal regenerated tail (arrowhead, less pigmented than the tail stump); bar, 1 mm. (b) histological section of ependymal tube inside the extra tail showing the central canal (arrowhead) and a CSFCN (arrow); bar, 10 μm. (c) Electron microscope view of the ependyma, detailing pale spaces among ependymal cells, the neural cell, a likely degenerating cell within the central canal (asterisk), and the limiting basement membrane (arrowhead); bar, 2 μm. (d) Detail on the narrow ependymal tanycytes containing bundles of intermediate filaments and separated by large electron-pale spaces; bar, 1 μm. (e) Higher magnification of pale spaces featuring thin axonal cross-sections (arrowheads), ependymo-glial sections (arrows), and larger glial sections (double arrows) present among ependymal tanycytes. Also degenerating material is seen; bar, 250 nm. (f) Pale and euchromatic neural cell that is close to a fascicle of nerves (axonal elongations); bar, 1 μm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neuron; deg, degenerating material; ep, ependyma; fi, filament bundles; ne, nerves (groups of regenerating axons) with glial processes present among ependymal cells; n, nucleus; nr, neuron

1964). Recent experimental studies have specifically suggested that Shh is among the main chondrogenic factors produced from the ependyma and/or its neural cell precursors, and that the release of Shh around the ependymal tube induces the differentiation of the cartilaginous tube (Lozito & Tuan, 2016). Further studies in vivo and in vitro on the regenerating ependyma and CSFCNs are, however, needed to provide further evidence on a possible stimulation of the process of regeneration, and presently the most likely function of CSFCNs in the regenerated SC appears to be mechanoreceptorial.

6 | CENTRAL CONNECTIONS AND A ROLE OF REGENERATED CSFCNs

As CSFCNs inside the regenerated lizard SC are isolated from the peripheral tissues by a continuous cartilaginous cylinder, their function is likely related to monitoring the movements and, perhaps, the biochemical composition of the CSF. As mechanoreceptors, these cells might be responding to the movements of the CSF and in particular the oscillations of the Reissner fiber inside the central canal when the tail moves (Alibardi, 1993; Alibardi & Meyer-Rochow, 1988, 1990). The mechanoreceptor function of CSFCNs has been recently demonstrated for the lamprey and zebrafish SC among tailed anamniotes (Bohm et al., 2016; Jalalvard et al., 2016).

The number of CSFCNs present in the regenerated tail SC varies from the species among lizard (3–10% generally), individuals and state of nutrition, but most likely from the mechanical activity of the tail. The latter hypothesis is supported by a behavioral experiment. In three tested species (*L. nigriplantare maccanni*, *L. delicata*, and *H. maculatus*) kept in active movement for 6–8 hr/day in about 3 weeks during tail regeneration, the number of formed CSFCNs increased slightly but significantly with respect to the normal regenerated tail of lizards kept in normal conditions, from an average of 17–19% CSFCNs in stimulated regenerated SC versus 12–13% CSFCNs present in normal regenerated SC (see fig. 2.10 in Alibardi, 2010).

The nervous connections between the normal SC and brain to the regenerated tail SC have been analyzed in detail in the chameleon lizard *A. carolinensis* (Duffy et al., 1992; Duffy, Simpson, Liebich, & Davis, 1990; Simpson & Duffy, 1994). Tract tracing studies using the retrograde tracers HRP and Lucifer yellow have indicated that CSFCNs in the regenerating SC are retrogradely labeled after application of the tracers to the SC relatively close to the regenerated SC or

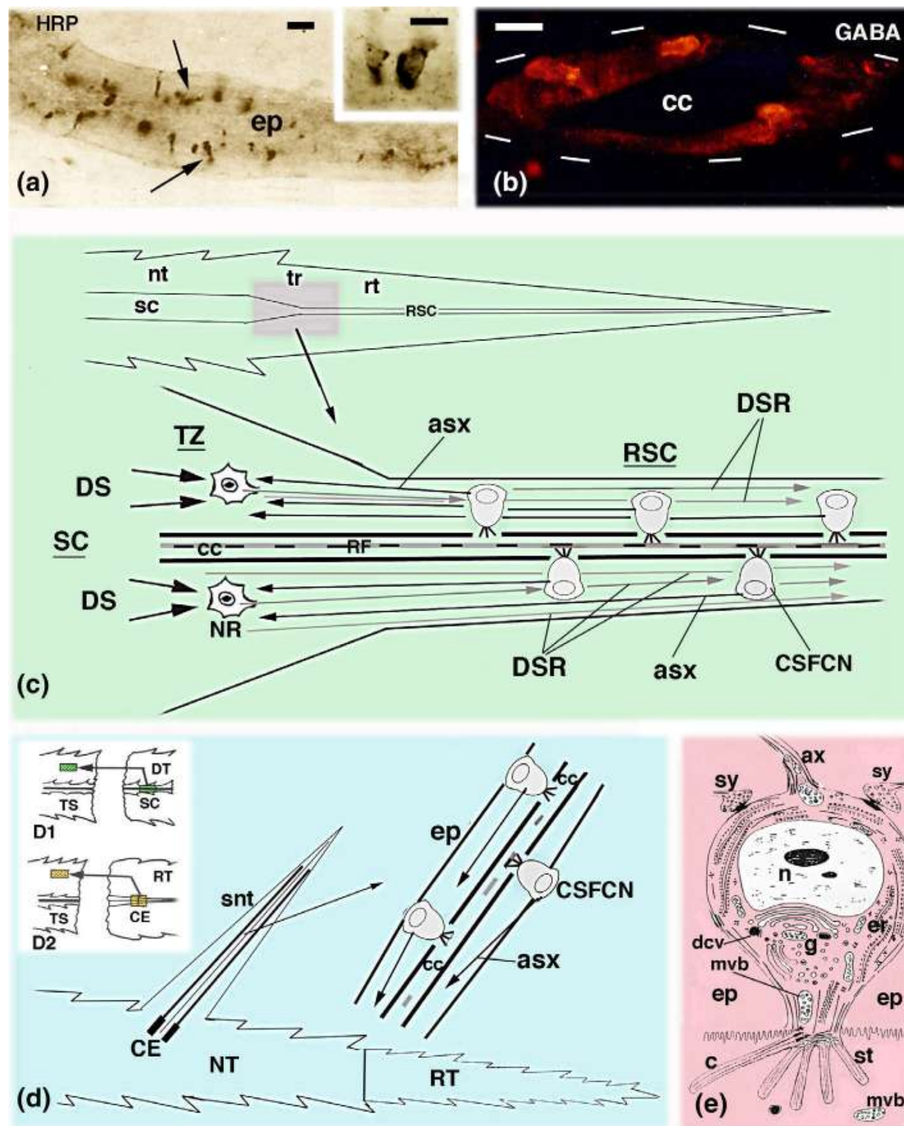

FIGURE 9 Microscopic images (a,b) and drawings showing the nervous circuits present in the regenerated SC (c,d). (a) Retrograde labeling of CSFCNs in *Anolis carolinensis* after HRP application in the lumbar SC. Various labeled cells (arrows) are seen along the ependyma isolated in toto (inset; bar, 10 μm). (b) Tetramethyl rhodamine isothiocyanate (RITC) GABA-immunofluorescence in ependymal (outlined by dashes) of *Podarcis muralis* showing three immunolabeled cells; bar, 10 μm. (c) Drawing of the simple neuronal network connections between the regenerated and normal tail SC. (d) Drawing illustrating the simple regenerated SC found inside supernumerary (additional) tail after autoimplant of SC (D1) or of cartilage-ependyma (D2). (e) Summarizing cytology of a CSFCN. asx, ascending axons from CSFCNs; ax, axon; c, cnidocilium; cc, central canal; CE, explanted/implanted cartilage-ependyma; CSFCNs, cerebrospinal fluid-contacting neuron; dcv, dense core vescicles; DS, descending (supraspinal) axons from the rostral SC/brain that forms synapses with neurons in the transition zone; DSR, descending axons from the transition zone; DT, distal tail stump; ep, ependyma; er, ergastoplasm; g, Golgi apparatus; n, nucleus; mvb, multivesicular body (exosome?); nt/NT, normal tail; NR, neurons of the transitional zone; RF, Reissner fiber (dashed); RSC, regenerated spinal cord; rt/RT, regenerated tail; SC, spinal cord; snt, supernumerary (additional) tail derived from autotransplant; st, stereocilia; sy, synaptic boutons; tr, transition region (passage from normal to regenerated SC); TS, proximal tail stump; TZ, transition zone of the SC

in the lumbar SC, but not in more rostral regions of the normal SC (Duffy et al., 1993; Figure 9a). Because CSFCNs present in the regenerated SC are GABA positive (Figures 1g, 5b, and 9b), it is possible that these cells give rise to prevalent inhibitory axons that reach the normal SC. Tract tracing studies have, however, shown that most ascending axons from regenerated CSFCNs terminate in the proximal SC stump connected to the regenerated cord (the "transition zone" according to Duffy et al., 1990, 1992, 1993; Simpson & Duffy, 1994). Long ascending projections (supraspinal pathways) from the regenerated cord to the normal SC and brain are therefore unlikely. Instead, some neurons localized in the transition zone that have received synaptic contacts from the regenerated SC (CSFCNs) are in contact with synaptic boutons derived from long descending axons from the thoracic SC and even from the rhombencephalon, especially the reticular formation and vestibular nuclei (Duffy et al., 1990, 1992). Therefore, there seem to be an indirect although limited connection

between the normal tail SC located above the point of amputation, the transition zone, the thoracic SC, and also the brain, but it is not known if these fibers carry vestibular inputs. The simple cell composition, neural connections between regenerated SC in normal and supernumerary tails obtained after SC/cartilage-ependyma (CE) implants, and the hypothesized function of the regenerated CSFCNs are summarized in Figure 9c–e.

7 | CSFCNs IN THE RECOVERED LUMBAR SC OF LIZARDS

The ability to generate CSFCNs in lizards appears not limited to the caudal SC, but seem to extend in more rostral regions of the SC. About 1 month after complete or partial transection of the lumbar SC in lizards, some recovery from hind-limb paralysis is obtained in a significant number of animals (Alibardi, 2014, 2015; Raffaelli & Palladini, 1969; Srivastava, 1992; Srivastava, Maheshwari, Tyagi, & Shaukat, 1994; Figure 10a). The transected SC forms a glial and connective scar indicated as “the bridge,” and localized between the proximal stump (located before the transection) and distal stump (located after the transection) of the injured SC (Figure 1a). However, some axons cross the bridge and reconnect the distal with the proximal SC stumps (Alibardi, 2014). Only few small neurons were retrogradely labeled in the proximal SC stump using the DiI tracer injected in the distal SC stump, suggesting that they may correspond to neurons of the intrinsic spinal “locomotor central pattern generator” of the SC, and that they might be responsible for the reactivation of hind limb movements in the initially paralyzed lizards.

The reactive ependymal cells of the injured proximal and distal stumps of the SC form terminal dilatations of the ependymal canal, or ependymal ampullae that can extend for some distance into the bridge. The ampullae, however, do not restore the continuity of the original central canal of the proximal with the distal stumps of the SC. Light microscopy and ultrastructural analyses reveal the presence of some CSFCNs after 30–45 days postoperation in the ependymal ampullae (Alibardi, 2014, 2015; Figure 10b–d). The derivation of these neurons, from local ependymal cells or from migrating neuronal precursors of the close SC stumps, has not been yet determined. As in the tail SC, also in the lumbar SC an intense cell proliferation of the ependymal

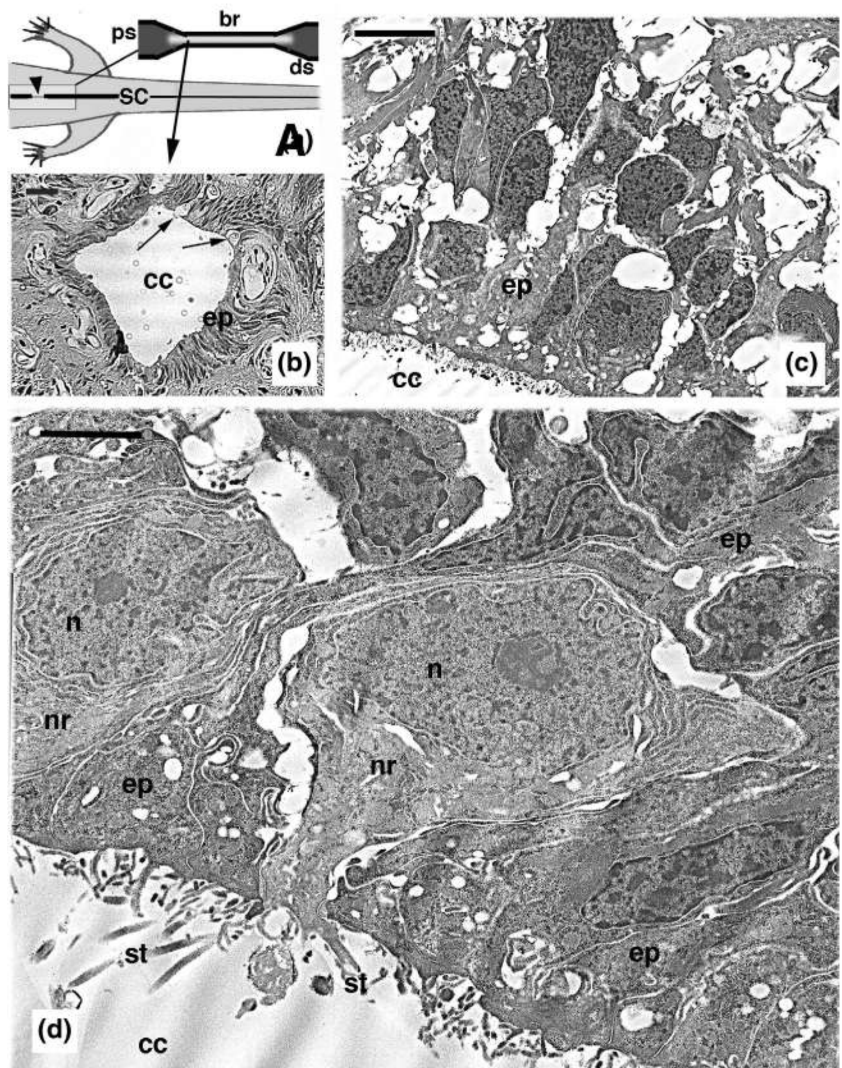

FIGURE 10 Schematic drawing illustrating the transection of the lumbar SC in lizard (a), the histology of the reactive central canal (b) and its ultrastructure (c,d) at 30 days postinjury. (a) The gap created by the transection (arrowhead) is enlarged to show the bridge where the central region in the proximal SC stump is shown in “b” (arrow). (b) Ependymal ampulla observed in cross section with a thick ependymal epithelium and two pale cells (arrows) contacting the enlarged central canal; bar, 20 μm. (c) Multilayered ependymal epithelium separated by numerous electron-pale spaces (largely empty); bar, 5 μm. (d) Detail on ependyma where two paler CSFCNs are seen; bar, 2 μm. br, bridge region; cc, central canal; CSFCNs, cerebrospinal fluid-contacting neuron; ds, distal stump of the SC; ep, ependyma; n, nucleus; nr, neuron; ps, proximal stump of the SC; SC, spinal cord; st, stereocilia

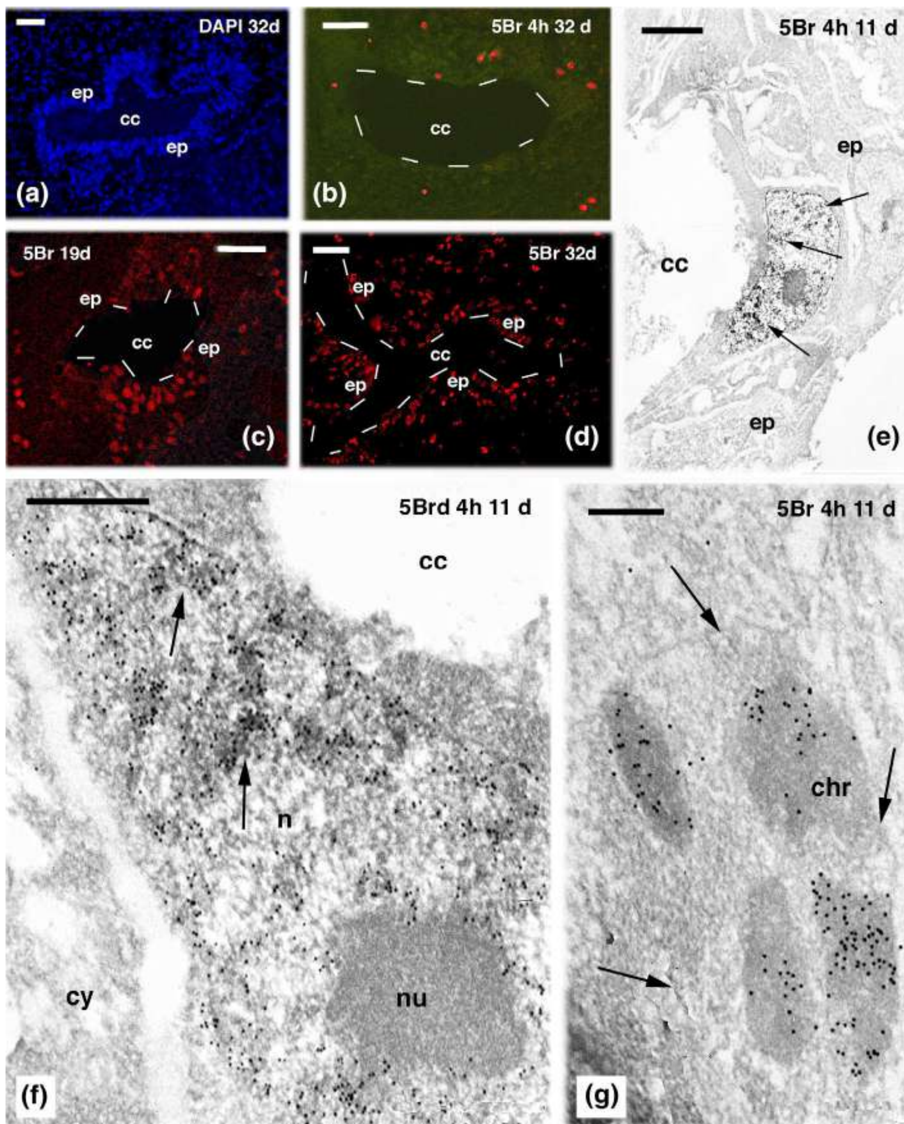

FIGURE 11 Immunolabeling for 5BrdU at progressive times (a–d, fluorescence; e–g immunogold) shows intense proliferation of ependyma in proximal stump of the SC. (a) 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining showing the enlarged ependymal canal at 32 days of regeneration; bar, 20 μm. (b) TRITC labeled nuclei in the enlarged ependymal canal at 32 days, 4 hr postinjection of 5BrdU; bar, 20 μm. (c) Labeled nuclei in the ependyma after 19 days of SC regeneration and 5BrdU injection that evidence labeling dilution; bar, 20 μm. (d) Labeled nuclei at 32 days of regeneration and 5BrdU injection to evidence the labeling dilution along the irregular ependymal canal; bar, 20 μm. (e) Labeled (arrows) nucleus 4 hr after injection of 5BrdU in an ependymal cell lining the central canal at 11 days of regeneration; bar, 2 μm. (f) Detail on the nuclear labeling (arrows) of the chromatin but not the nucleolus, 4 hr postinjection of 5BrdU; bar, 1 μm. (g) Labeling dilution observed in chromosomes of a dividing ependymal cell (arrows point to the remnants of the nuclear membrane); bar, 0.5 μm. cc, central canal; chr, chromosomes; cy, cytoplasm; ep, ependymal epithelium/cell; n, nucleus; nu, nucleolus

epithelium occurs, and also numerous glial cells are proliferating at 11–32 days postinjury, as shown by 5BrdU-labeling at progressive periods from the injection (Figure 11). Although ependymal cells appears to proliferate and dilute the label through cell division (Figure 11c,d,g), no labeled CSFCNs are present in these ampullae at 22 days posttransection. Therefore, at the present time although CSFCNs are present in the ependymal canal of the two interrupted lumbar SC stumps at 30–45 days postamputation, their possible derivation from the reactive ependyma of the dilated ampullae remains uncertain.

In conclusion, the study on the regenerating tail and lumbar SC of lizards shows that (a) some ependymal cells of the tail SC have neurogenic potential and give rise to CSFCNs and (b) some of the axons present in the regenerated SC are ascending and derive from the CSFCNs. Other axons instead descend from the SC closer to the regenerated one. Figure 9c–e schematically summarizes the connections and cytological characteristics of CSCFNs in the normal regenerated tail (Figure 9c) and in supernumerary (Figure 9d) tails of lizards. The ultrastructural, experimental, and tract tracing studies mentioned above show that although simplified in comparison to the original SC and isolated within the cartilaginous tube, the regenerated SC of lizards forms few neurons that likely monitor the movement/composition of the CSF during tail movements. It has been hypothesized that CSFCNs help the animals to maintain their balance during running, sprinting, and climbing (anole lizards and geckos). The possibility that CSFCNs are regenerated in other areas of the SC and even in the brain during normal physiologic renewal or traumatic events remains to be shown in other amniotes, including mammals.

8 | CSFCNs REGENERATION IN OTHER CAUDATE TETRAPODS

The detection of CSFCNs in the caudal SC of lizards stimulated the comparative ultrastructural survey on other vertebrates capable of

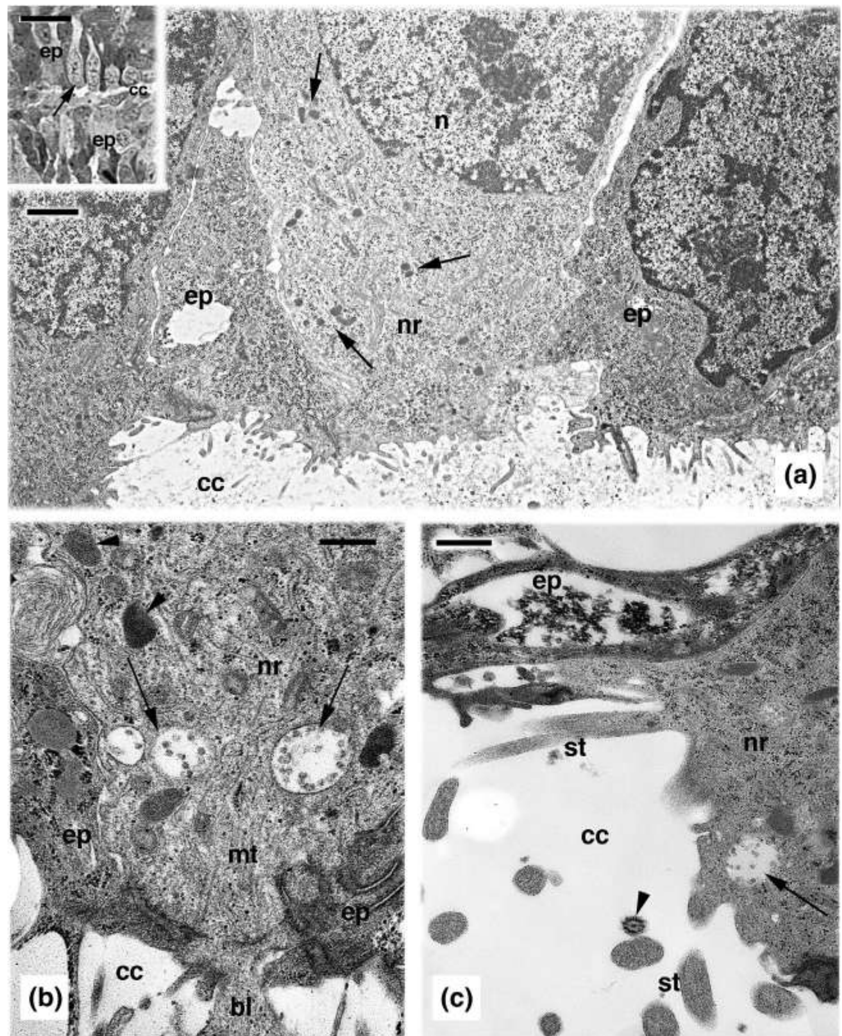

FIGURE 12 Ultrastructure of regenerated CSFCNs in *Sphenodon punctatus* caudal SC. (a) Some pale cells observed among ependymal cells at 5 months of tail regeneration (arrow in the inset; bar, 10 μm) and containing sparse secretory granules (arrows) differentiate into CSFCNs; bar, 1 μm. (b) Luminal cytoplasmic blebs protrude in the central canal and numerous secretory granules (arrowheads) and multivesicular bodies (arrows) are commonly present together fascicles of microtubules); bar, 0.5 μm. (c) In old regenerated SC, 10 months or older, tufts of stereocilia are present together a cnidocilium (arrowhead). The arrow indicates a multivesicular body; bar, 0.5 μm. bl, cytoplasmic bleb; cc, central canal; CSFCNs, cerebrospinal fluid-contacting neuron; ep, ependyma; mt, microtubules; n, nucleus; nr, neuron; st, stereocilia

tail regeneration. These studies have also shown that CSFCNs can be formed within the regenerating caudal SC in the ancient lizard-like reptiles tuatara (*S. punctatus*), and in some urodele and anuran amphibians. In the tuatara, the process of tail regeneration is slow, and tanycytes in the regenerated SC appear around 5 months after the amputation of the tail (Alibardi & Meyer-Rochow, 1990). Like in lizards CSFCNs are electron-paler cells containing sparse secretory vesicles, large dense granules, dense core vesicles of 90–150 nm in diameter, and multivescicular bodies that have probably been extruded into the CSF (exosomes?; Figure 12b,c). In regenerated tails of 5 months or older, CSFCNs feature groups of stereocilia inside the central canal (Figure 12c). Synaptic boutons are occasionally seen on the external (nonluminal) side of their cell body, facing the basement membrane. These cells are detected in old regenerated tails, probably over 2–3 years old after the beginning of tail regeneration.

During tail regeneration in newts (*T. cristatus* and *T. vulgaris*), the regenerating ependyma gives rise to numerous neurons after 20–30 days from amputation (Figure 13a). The regenerated tail is about 8–15 mm in length at 40 days of regeneration, and is effectively used for swimming. The progressive stages of differentiation from elongated and denser ependymal cells to electron-paler and round CSFCNs take about 1 month but it is completed at 2 months of tail regeneration (Alibardi, 1989). The Golgi apparatus of these cells produces dense core vesicles of peptidergic type, 90–150 nm in diameter, that are mainly observed in the peripheral axon directed among the forming white matter of the regenerated SC. Pale cells, mainly formed among ventral ependymal cells, are completely differentiated into CSFCNs when also synaptic boutons and long stereocilia are established around 2 months of tail regeneration (Figure 13b,c). Most of the boutons contain a mix of small round and pleomorphic vesicles, and also of glycogen particles in boutons seen in these cells at 1–2 months of regeneration (Figure 13c).

Also in the tadpoles of the African toad, *X. laevis*, in addition to other types of neurons (Filoni & Bosco, 1981), the differentiation of CSFCNs takes place in about 30–40 days of tail regeneration, mainly from ependymal cells localized in the ventral-lateral side of the regenerating SC (Alibardi, 1990, 1990–1991). Larvae of anurans are known to regenerate some types of neurons, including sensitive Rohon-Beard neurons, located in the dorsal-lateral side of the new SC

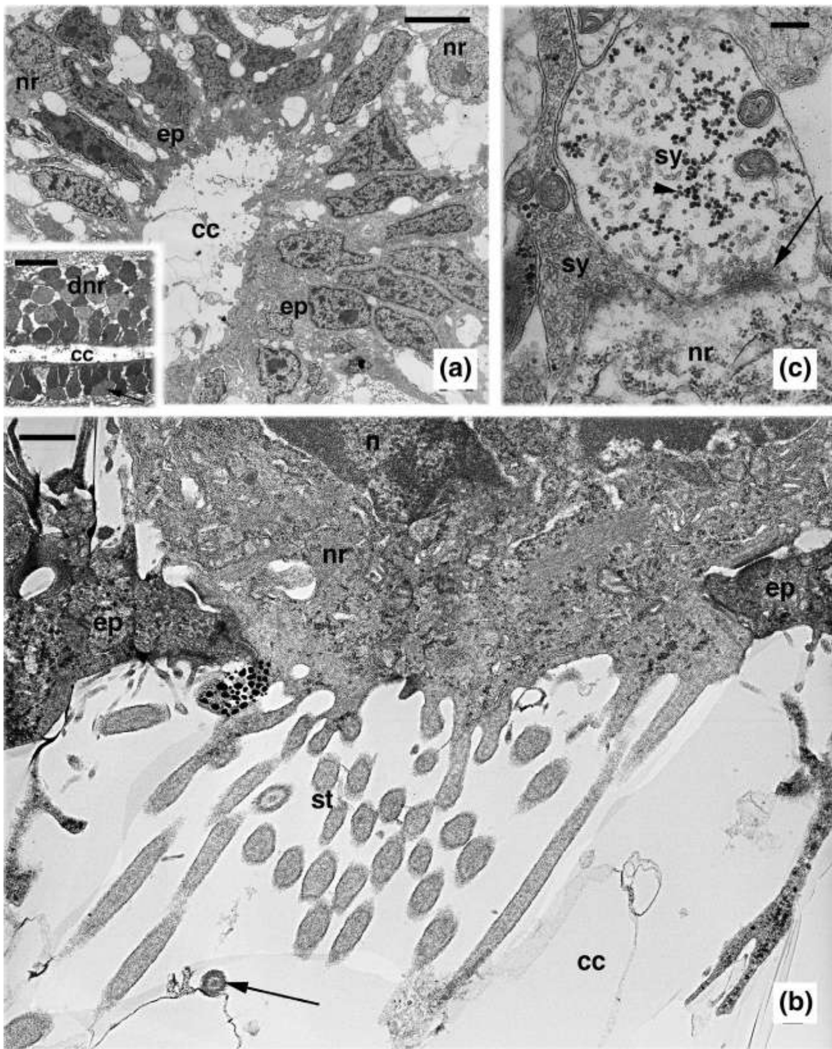

FIGURE 13 Ultrastructure of the regenerating tail SC in *Triturus cristatus* 1 month postamputation. (a) Cross-section showing the presence of peripheral and paler neural cells more in the ependymal epithelium. The inset (bar, 10 μm) shows a detail of the stratification of the SC in longitudinal section, including pale neurons (arrow) in the less stratified ventral SC, from where CSFCNs are derived; bar, 5 μm. (b) Stereocilia on the luminal surface of a differentiating CSFCN (the arrow indicates a cnidocilium); bar, 0.5 μm. (c) Synaptic boutons containing pleomorphic and flat vesicles, together glycogen granules (arrowhead), in contact (arrow) with a CSFCN; bar, 200 nm. cc, central canal; CSFCNs, cerebrospinal fluid-contacting neuron; ep, ependyma; nr, neuron; st, stereocilia; sy, synaptic bouton

(Filoni & Bosco, 1981). This capability is progressively lost as metamorphosis approaches and the tail is resorbed, indicating that these ventral and dorsal neurons are involved in swimming movements. In *X. laevis* and *R. dalmatina*, the length of the regenerating tail after 30–40 days from the amputation varies from 6 to 10 mm and is similar to the original one, although less pigmented, and is quite effective in swimming, like the original one. From the initial mono-layered ependyma, few paler and pear-like neurons are present at 20–30 days of tail regeneration (Figure 14a,b). The ultrastructural analysis shows that the electron-pale cytoplasm of some of these cells, especially in the ventro-lateral region of the regenerated SC, contains sparse large dense core vesicles of peptidergic type derived from the Golgi apparatus. Well-developed stereocilia are present in the lumen at 30–40 days of regeneration, together with a single cilium (Figure 14b, c). Few axosomatic synaptic boutons are detected on the cell body at 1 month of tail regeneration, storing pleomorphic vesicles and often also small (50–60 nm in diameter) dense core vesicles (Figure 14d). It is likely that, like for the other neurons, CSFCNs are also not regenerated at stages close to metamorphosis, when the tail becomes rapidly resorbed (Filoni & Bosco, 1981).

In conclusion, CSFCNs of the SC of the tail appear in the first 1–2 months of caudal regeneration of lizards and amphibians, suggesting that these cells are essential for the role of the tail in the general physiology of motion in caudate tetrapods. Recent anatomical, physiological, and cell biology studies on zebrafish (Bohm et al., 2016; Fidelin et al., 2015; Orts-Del'Immagine & Wyart, 2017; Wyart et al., 2009) and lamprey (Jalalvard et al., 2014, 2016) have indeed shown that CSFCNs are intraspinal mechanoreceptors sensitive to the bending movements and intervene in the modulation of activity of the "locomotor central pattern generator" during swimming. Their regeneration is therefore important for the coordination and the movement associated with the tail in caudate vertebrates. Another function of these cells, especially in the hypothalamus, is related to sensing changes in the pH of the CSF (Jalalvard et al., 2016; Orts-Del'-Immagine & Wyart, 2017). The functions of CSFCNs in the brain regions were they are mainly concentrated, the pavement walls of the fourth ventricle and Silvio aqueduct, remains largely unknown, but the establishment of a CSF-contacting nucleus knockout rat model (Song & Zhang, 2018) will likely provide further clues on their roles in future studies.

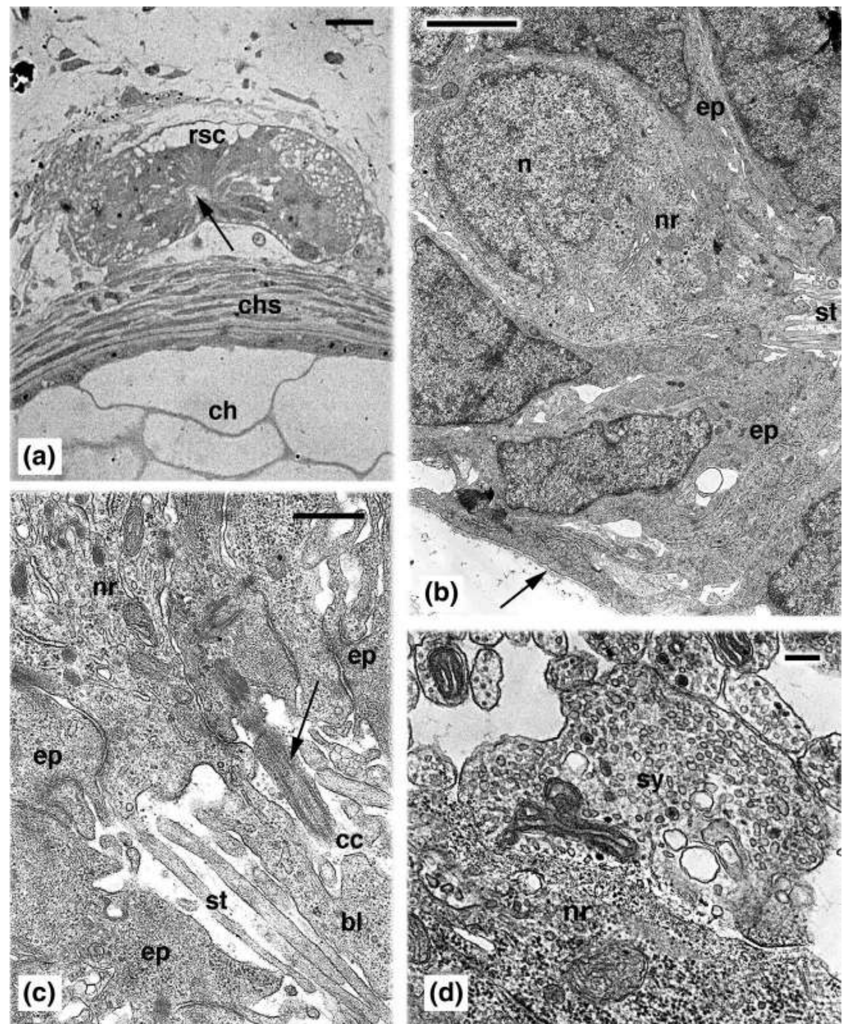

FIGURE 14 Ultrastructure of regenerating tail SC of *Xenopus laevis* 1-month after tail amputation. (a) Light microscopy image of cross-sectioned notochord with the new SC (the arrow points to the central canal); bar, 10 μm. (b) Electron-pale CSFCN among ependymal cells. The arrow indicates the limiting basement membrane; bar, 2 μm. (c) Detail on the stereocilia and cilium (arrow) within the lumen; bar, 0.5 μm. (d) Forming axosomatic bouton (no presynaptic and postsynaptic thickenings are clearly visible yet) on a CSFCN; bar, 200 nm. bl, cytoplasmic bleb; cc, central canal; ch, notochord; chs, notochord sheath; CSFCNs, cerebrospinal fluid-contacting neuron; ep, ependyma; n, nucleus; nr, neuron; rsc, regenerating spinal cord; st, sterocilia; sy, synaptic bouton

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

ORCID

Lorenzo Alibardi https://orcid.org/0000-0002-8247-2217

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