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Organization of Glial Cells in the Adult Sea Cucumber Central Nervous System

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KEY WORDS
echinodermata; holothurian; cell division; cell death; glia/neuron ratio; radial glia

ABSTRACT
The nervous system of echinoderms has long been considered too unique to be directly comparable to the nervous system of other Deuterostomia. Using two novel monoclonal antibodies in combination with epifluorescence, confocal, and electron microscopy, we demonstrate here that the central nervous system of the sea cucumber *Holothuria glaberrima* possesses a major non-neuronal cell type, which shares striking similarities with the radial glia of chordates. The basic features in common include (a) an elongated shape, (b) long radial processes, (c) short lateral protrusions branching off the main processes and penetrating into the surrounding neuropile, (d) prominent orderly oriented bundles of intermediate filaments, and (e) ability to produce Reissner's substance. Radial glia account for the majority of glia cells in echinoderms and constitutes more than half of the total cell population in the radial nerve cord and about 45% in the circumoral nerve ring. The difference in glia cell number between those regions is significant, suggesting structural specialization within the seemingly simple echinoderm nervous system. Both cell death and proliferation are seen under normal physiological conditions. Although both glia and neurons undergo apoptosis, most of the mitotic cells are identified as radial glia, indicating a key role of this cell type in cell turnover in the nervous system. A hypothesis is proposed that the radial glia could be an ancestral feature of the deuterostome nervous system, and the origin of this cell type might have predated the diversification of the Chordata and Ambulacraria lineages.

INTRODUCTION

Glial cells are known to be a crucial component of the nervous tissue required for its development, maintenance, and function (Allen and Barres, 2009; Kettenmann and Verkhratsky, 2008). It has been proposed that the evolution of the central nervous system (CNS) could have been driven in large part by changes in glia organization (Giaume et al., 2007; Oberheim et al., 2006). Moreover, a significant number of nervous system diseases are to a great extent due to glial pathology (Giaume et al., 2007; Tian et al., 2005). Therefore, detailed knowledge of glial biology is necessary to understand virtually any phenomenon in the nervous system. Organization and function of glial cells has been particularly well studied in vertebrates and, to some extent, in arthropods (Awasaki et al., 2008; Gocht et al., 2009; Sullivan et al., 2007), but remains largely uncharacterized in other taxa. Among invertebrates, echinoderms deserve particular attention, since they constitute a nonchordate deuterostome group with a centralized nervous system and are therefore essential for understanding the evolution, development, and function of the deuterostome CNS, which cannot be clarified by studying vertebrates alone.

Among echinoderms, the larval nervous system has been studied in great detail (e.g., Byrne et al., 2007; Elia et al., 2009; Nakano et al., 2006), whereas the nervous system of adults has received surprisingly much less attention. The progress in echinoderm neurobiology has been seriously hampered by two issues. First, almost all studies on echinoderm nervous system published so far dealt with characterization of various neuronal populations. On the other hand, data on the echinoderm glia are scarce and largely incomplete, since accounts focusing specifically on this cell type have been a rare exception (Bargmann et al., 1962; Mashanov et al., 2009; Viehweg et al., 1998). The second issue is the lack of reliable immunocytochemical techniques that would allow unambiguous discrimination between neuronal and glial cells in the echinoderm nervous tissue. In vertebrates, glial cells in the CNS are usually detected using an established set of antibodies including those to the glia fibrillary acid protein (GFAP), vimentin, nestin, glutamine synthetase, and S100β (Misson et al., 1988; Sofroniew and Vinters, 2010). We have been unable to obtain unambiguous immunostaining of the holothurian nervous tissue with a variety of commercially available antisera to the "standard" glial markers (see Materials and Methods). It has been shown, however, that the non-neuronal cells of the echinoderm nervous system are capable of producing the so-called Reissner's substance (Mashanov et al., 2009; Viehweg et al., 1998), which in vertebrates is known to be secreted by a phylo-

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genetically conserved secretory radial glia cell subtype of the subcommissural organ and also by the radial glia of the floor plate in embryogenesis (Lichtenfeld et al., 1999; Oksche, 1961). Unfortunately, the secretory nature of Reissner's substance makes it unsuitable as a glia-specific marker if a single-cell resolution is needed. In most preparations of the holothurian and asteroid CNS, the strongest immunolabeling is produced by the extracellular material that has been released from the cells to the apical surface of the neuroepithelium (Mashanov et al., 2009; Viehweg et al., 1998). So far, unequivocal identification of glial cells in echinoderms has been only possible by transmission electron microscopy, since the only reliable distinguishing feature of this cell type has been their characteristic shape and the presence of long bundles of intermediate filaments in the cytoplasm (Bargmann et al., 1962; Mashanov et al., 2006, 2008, 2009; Märkel and Röser, 1991; Viehweg et al., 1998). Thus, any quantification or any study employing co-localization of different markers have been highly challenging because of the amount of time and labor involved in specimen preparation for electron microscopy.

The aim of this study was to generate monoclonal antibodies specifically recognizing echinoderm glial cells and to use them as a tool to characterize the basic parameters of the glial population in the sea cucumber CNS, including cell shape, distribution, relative abundance, and cell death/proliferation balance. This study lays out a foundation for further research involving echinoderm glial cells.

MATERIALS AND METHODS

Antibodies

Adult individuals of the sea cucumber *Holothuria glaberrima* Selenka, 1867 were anesthetized in 0.2% 1,1,1-trichloro-2-methyl-2-propanol hydrate (Sigma) in seawater for 15 min at room temperature. The radial complexes (including the radial nerve cord, water-vascular canal, hemal lacuna, and the medial region of the longitudinal muscle band) were separated from the surrounding tissues and homogenized in ice-cold extraction buffer (100 mg wet weight/mL buffer) containing 1% Triton X-100, 100 mM MES, 1 mM EGTA, 0.5 mM $MgSO_4$, 0.5 mM PMSF, and 0.1% protease inhibitor cocktail (Sigma), pH 7.0 (Merrick et al., 1995). The tissue homogenate was kept on ice for 15 min and then centrifuged at 20,000*g* for 2 h at 4°C. The supernatant was discarded, and the pellet was rehomogenized in 10 mM PBS (pH 7.4). After an additional centrifugation at 20,000*g* for 2 h at 4°C, the pellet was again rehomogenized in PBS and processed for immunization and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For immunization, the extract was emulsified in equal volume of TiterMax Gold Adjuvant (Sigma), and ~50 μg of total protein in 100 μL of suspension was injected intraperitoneally in two female Balb/C mice. The second injection was performed 3 weeks later. On day 42 (after the initial injection), the animals were injected with a similar amount of the protein without the adjuvant. On day 45, the spleens were processed for fusion to Sp20-Ag14 myeloma cells (ATCC CRL-1581). Monoclonal antibodies that recognized sea cucumber glial cells were identified by immmunohistochemistry. For other antibodies used in this study, see Table 1.

Immunoblots

The radial nerve extract was mixed with 0.5 vol of SDS gel loading buffer (150 mM Tris, 200 mM DTT, 6% SDS, 0.3% Bromophenol blue, 30% glycerol, pH 6.8), separated by SDS-PAGE and transferred to Immun-Blot PVDF Membrane (Bio-Rad). The blots were blocked in 5% nonfat milk in PBS with 0.1% Tween (PBST), incubated in primary antibodies diluted in PBST overnight at 4°C, washed, incubated in peroxidase-linked anti-mouse antibodies (1:10,000, GE Healthcare, NXA 931), and detected by chemiluminescence using ECL Plus Western Blotting Detection Reagents (Amersham, RPN2132).

Immunolocalization, Cell Death Detection, and Cell Proliferation Assay

Tissue processing for immunofluorescence and immunoelectron microscopy, including fixation, embedding, and labeling, was performed as described elsewhere (Mashanov et al., 2009). In brief, the tissues were fixed overnight with 4% paraformaldhyde in 0.01 M PBS. After washing in the buffer and cryoprotection in buffered sucrose solutions, the samples were frozen and cryosections (10 μm) were cut with a Leica CM1850 cryostat. The primary antibodies were applied overnight at 4°C. Incubation in the secondary antibodies was performed at room temperature for 1 h.

To localize the antigen at the ultrastructural level, we used the pre-embedding immunogold technique. Cryosections were preincubated in 0.05 M buffered glycine followed by 0.1% BSA-c™ (Aurion). The primary antibodies were applied overnight at 4°C. The sections were then incubated in a gold-conjugated goat-antimouse antibody (the average gold cluster diameter below 0.8 nm; Aurion). To facilitate observation of ultrasmall gold clusters, silver enhancement reagents R-Gent SE-EM and R-Gent SE-LM (Aurion) were used. The sections were then dehydrated in ethanol and embedded in Araldite (EMS). Ultrathin sections were cut using a Leica EM UC6 ultramicrotome, collected on grids, and slightly counterstained with saturated uranyl acetate in methanol.

Cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem). Proliferating cells were labeled by injecting animals into the coelomic cavity with 5-bromo-2-deoxyuridine (BrdU). BrdU (~0.1 mg per animal) was injected daily for periods ranging from 1 to 7

TABLE 1. Antibodies Used in This Study

Antibodies used	Source	Host species	Dilution	Result
3CB2 (vimentin)	Developmental Studies Hybridoma Bank	Mouse	Undiluted supernatant	−
40E-C (vimentin)	Developmental Studies Hybridoma Bank	Mouse	Undiluted supernatant	−
AFRU	Dr. J.M. Grondona (University of Malaga, Spain)	Rabbit	1:1,000–1:4,000	+
BrdU (clone BU-1)	Amersham (RPN202)	Mouse	1:500–1:1,000	+
BrdU	GenWay (20-783-71418)	Rat	1:400	+
Calbindin	Abcam (ab1126)	Rabbit	1:2,500–1:5,000	+
ERG1	This study	Mouse	1:1–1:7	+
ERG2	This study	Mouse	1:30	+
GABA	Sigma (A2052)	Rabbit	1:1,000–1:2,000	+
GFAP	Chemicon (AB1540)	Rabbit	1:50	−
GFAP	Sigma (G9269)	Rabbit	1:20–1:500	−
GFSKLYFamide	Diaz-Miranda et al., 1995	Rabbit	1:500–1:1,000	+
Glutamine synthetase	Sigma (G2781)	Rabbit	1:50–1:1,000	+
Nurr1/Nur77 (E-20)	Santa Cruz Biotechnology (SC-990)	Rabbit	1:250–1:1,000	+
Parvalbumin	Affinity BioReagents (PA1-933)	Rabbit	0.2 μg/mL	+
S100	Zymed (18–0046)	Rabbit	1:100–1:400	−

+, works on *H. glaberrima* nervous tissue; −, does not work on *H. glaberrima* nervous tissue.

days. The pieces of the radial nerve cord were fixed and processed for BrdU immunohistochemistry.

Immunofluorescence preparations were viewed and photographed using either an epifluorescent Nikon Eclipse 600 microscope or a Zeiss 510 Meta confocal laser scanning microscope. Optical sections for confocal series were taken at intervals of $\leq$1 μm. For visualization purposes, two-dimensional projection images were produced using the public domain Fiji software (http://pacific.mpi-cbg.de). Preparations for immunoelectron microscopy were analyzed and photographed using a LEO 922 OMEGA transmission electron microscope.

Cell Counting

Cells were counted on panoramic micrographs of the whole cross-sectioned profiles of the radial nerve cord or the nerve ring taken with an epifluorescent microscope, equipped with a 40× objective. If cells were packed tightly, additional images were taken at higher magnification and resolution using an oil immersion 100× objective to discriminate between the adjacent cells. At least three animals were used per experimental group. Counting was performed on three to five sections per animal. Student's unpaired two-tailed test was used for statistical analysis.

RESULTS

Characterization of Novel Monoclonal Antibodies: Morphological Features of Radial Glial Cells

Immunization of mice with the extract of the holothurian radial nerve (see Fig. 1 for introduction to basic neuroanatomy of sea cucumbers) yielded 18 antibody-producing clones. Glia-specific antibodies were chosen as those that specifically labeled only cells with glia-like morphology in cross sections of the radial nerve cord. Two of them, which we designated as ERG1 and ERG2, were chosen for this study. The two antibodies displayed different patterns on Western blots of the radial nerve cord extract. The ERG1 antibody labeled a single band at ~300 kDa, whereas the ERG2 antibody recognized two major bands at ~100 and 250 kDa and a minor band at ~200 kDa (see Fig. 2). The different nature of the epitopes recognized by the antibodies was also further confirmed by the difference in response of the antigens to certain forms of pretreatment. For instance, tissue sections lose immunoreactivity for ERG1, but not for the ERG2 antibody after being digested with proteinase K (7.5 μg/mL PBS, 10 min, room temperature). In contrast, after an additional postfixation of cryosections in paraformaldehyde vapors (1 h, room temperature), the ERG2 antibody failed to produce any staining, whereas ERG1 showed the usual labeling properties (data not shown).

The immunofluorescent labeling with the ERG1 and ERG2 antibodies yielded a comprehensive way of visualizing the glial architecture in the holothurian CNS (see Fig. 3), in such detail and completeness that has never been achieved by using routine histological stains or TEM. Although the two antibodies obviously recognized different antigens, they both specifically labeled the same cell type, namely non-neuronal cells in the neuroepithelia of the radial nerve cord and the nerve ring (Figs. 3–5). Morphologically, the immunopositive cells turned out to be very similar to the radial glia in chordates (see below; see Fig. 3); therefore, we use the term "radial glia" to designate them in this article. Since echinoderm glial cells (also known as support cells, or Stützzellen) have been previously identified using ultrastructural criteria only, we implemented immunogold approach (see Fig. 4) to confirm that the immunoreactive cells show the morphological characteristics of the echinoderm glia (Bargmann et al., 1962; Mashanov et al., 2006, 2008, 2009; Märkel and Röser, 1991; Viehweg et al., 1998). All labeled cells had a long process and contained prominent bundles of intermediate bundles in their cytoplasm, i.e., showed cytological signs of glial nature (see Fig. 4). The glial processes form a dense structural framework of the neuroepithelium enmeshing the neuropile; sometimes, the processes of adjacent cells are organized in fascicles (see Fig. 3). No labeling of cells of neuronal morphology or surrounding neuropile was observed. Conversely, we saw no unlabeled cells that we could define as glial based on the morphological criteria. At the subcellular level, the immunogold labeling is

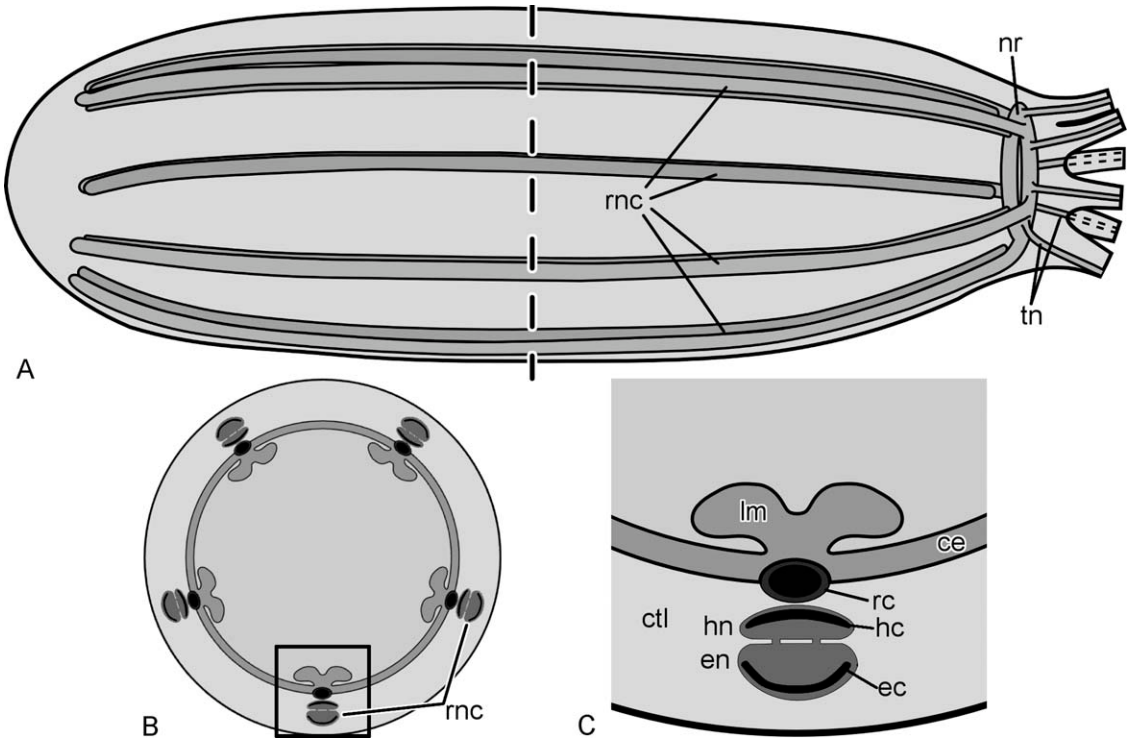

Fig. 1. Diagram showing anatomical organization of the sea cucumber central nervous system. (**A**) Lateral view of the adult sea cucumber, oral end to the right. (**B**) Cross section at the mid-body level (dashed line in A). (**C**) Higher magnification of the boxed area in B. The major components of the nervous system in echinoderms are the circumoral nerve ring (nr) and the radial nerve cords (rnc). The five radial nerve cords run from the anterior to the posterior end of the body within the inner connective tissue (ctl) of the body wall. Each of the radial nerve cords is composed of interconnected ectoneural (en) and hyponeural (hn) bands. At the anterior end of the body, the ectoneural bands are connected to the nerve ring. In sea cucumbers, both the ectoneural and the hyponeural parts of the nervous system contain cavities called the epineural (ec) and the hyponeural (hc) canals, respectively. The inner wall of the epineural canal and the outer wall of the hyponeural canal are thickened to form a tall neuroepithelium. The opposite sides of the canals are overlaid by flattened roof epithelia. ce, coelomic epithelium; ctl, connective tissue layer of the body wall; ec, epineural canal; en, ectoneural part of the radial nerve cord; hc, hyponeural canal; hn, hyponeural part of the raidial nerve cord; lm, longitudinal muscle; nr, circumoral nerve ring; rc, radial canal of the water-vascular system; rnc, radial nerve cord; tn, tentacular nerve. Figure not to scale. (From Mashanov et al., Front Zool, 2009, 6, 11, ©BioMed Central, reproduced by permission.)

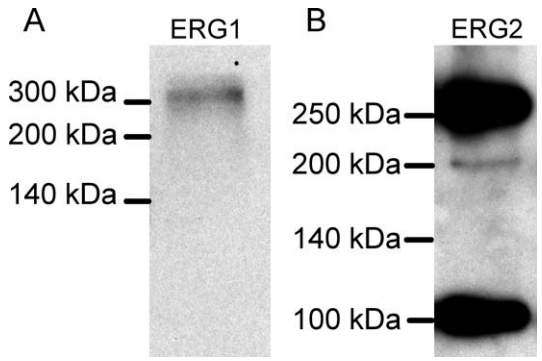

Fig. 2. Immunoblot with the novel ERG1 and ERG2 monoclonal antisera. Both lanes are the extract of the radial nerve cord of the sea cucumber *H. glaberrima*.

almost exclusively associated with the cell surface or the subsurface region and does not occur in the inner areas of the cytoplasm. The noteworthy difference between the staining patterns produced by the two antibodies is that ERG1 uniformly labels the whole cell (Figs. 3A,C,D and 4A,B,D), while ERG2 produces strong immunostaining of the glial cell somata (and occasionally of the basal endfeet), and much weaker (although still reliably discernible) staining of the processes (see below; Figs. 3B,E,F and 4E,F). Most of the immunopositive radial glial cells show unipolar morphology, with their somata forming a dense layer in the apical region and slender basal processes spanning the whole height of the neural parenchyma (Fig. 3A,C–F) to terminate with a cone-shaped endfoot at the basal lamina (Fig. 4D). The basal processes occasionally bifurcate (Figs. 3E,F and 4C) and often bear small lateral protrusions branching off the main processes (Figs. 3E,F and 4F). Interestingly, intermediate filaments are entirely absent from those fine branches (Fig. 4F). Some of the immunopositive cells have their cell bodies submerged into the neural parenchyma at various depths (Fig. 3D,F, insets). These cells display bipolar organization with two processes directed toward the apical and the basal surfaces of the neuroepithelium, respectively, but show otherwise similar morphological characteristics as the unipolar glial cells.

Co-localization Analysis

To characterize the immunophenotype of the ERG1/ERG2-immunopositive cells and further confirm their

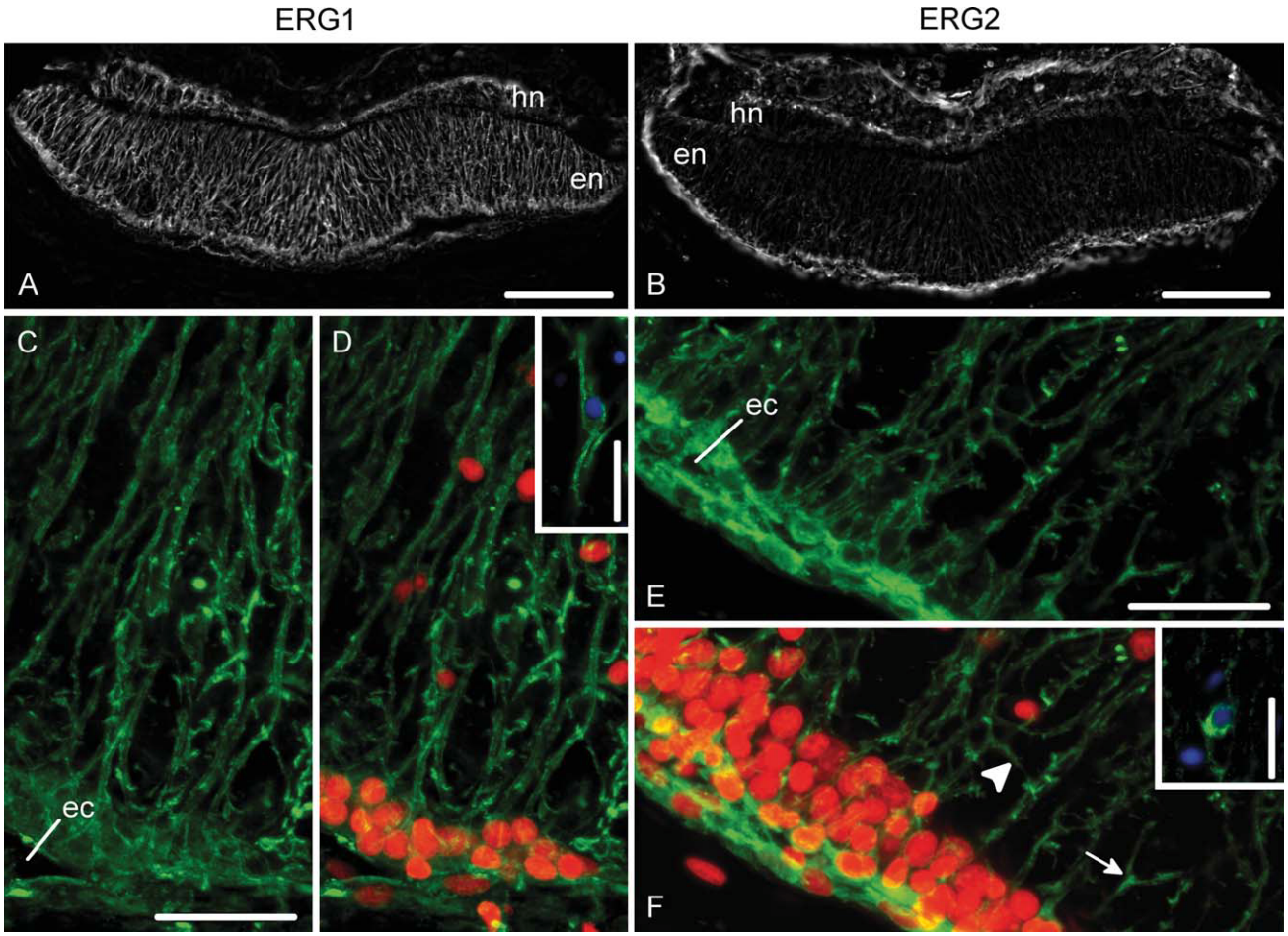

Fig. 3. Immunofluorescent staining of cross sections of the radial nerve cord with the ERG1 (**A**,**C**,**D**) and EGR2 (**B**,**E**,**F**) monoclonal antibodies. (A,B) Low-magnification view of the cross-sectioned radial nerve cord immunostained with the ERG1 and ERG2 antibodies, respectively. (C) Confocal image of ERG1-immunopositive glial cells in the ectoneural band of the radial nerve cord. (D) The same as (C) but also showing nuclei stained with propidium iodide. The inset in (D) shows an ERG1-positive glial cell body deeply submerged into the neural parenchyma; epifluorescence microscopy with nuclei counterstained with Hoechst. (E,F) Confocal images of glial cells in the ectoneural part of the radial nerve, showing ERG2 immunoreactivity alone (E) or combined with propidium iodide nuclear staining (F). Note the branching glial processes (arrow) and short lateral protrusions (arrowhead) branching off the main processes. The inset in (E) shows an ERG2-immunoreactive cell body in the neural parenchyma; epifluorescence microscopy with Hoechst nuclear staining. ec, epineural canal; en, ectoneural part of the radial nerve cord; hn, hyponeural part of the radial nerve cord. Scale bars: (A,B) 100 μm, (C–F) 20 μm.

glial identity, we performed double immunofluorescent labeling with our novel monoclonal antisera and a series of other glial and neuronal markers (see Fig. 5). Unfortunately, most of the available "traditional" glial markers failed to work on the sea cucumber nervous tissue in spite of various tissue fixation and antigen retrieval techniques applied in cases, when the routine protocol (see Materials and Methods) did not produce reliable staining. Out of the panel of glia-specific antibodies, only the anti-Reissner's substance antiserum (AFRU) showed a reliable and consistent labeling pattern. In agreement with our earlier data (Mashanov et al., 2009), the Reissner's substance-like material mostly seemed to form extracellular cap-like deposits on top of unipolar ERG1/ERG2-immunopositive cells, whose cell body reaches the apical surface of the neuroepithelium (Fig. 5A–A"). The AFRU antiserum also labeled rare singly scattered cell bodies within the neural parenchyma, which showed no immunoreactivity with either ERG1 or ERG2 (Supp. Info. Fig. 1). We saw no co-localization of labeling patterns between the novel antisera ERG1 and ERG2 and neuronal markers (Fig. 5B–D"), including antibodies directed against the neurotransmitter GABA, the neuropeptide GFSKLYFamide, and the dopaminergic neuron-specific transcription factor Nurr1. Some of the ERG1/ERG2-immunopositive cells were labeled with anti-calbindin D28k antibody (Fig. 5E–E"), which also seemed to label neuronal cell bodies and processes (data not shown), but not with an antiserum directed against another calcium-binding protein, parvalbumin (Fig. 5F–F").

Quantitative Analysis of Glia

We applied the ERG1 and ERG2 antibodies as specific glial markers to estimate the relative abundance of glia in the radial nerve cord and the nerve ring of the sea

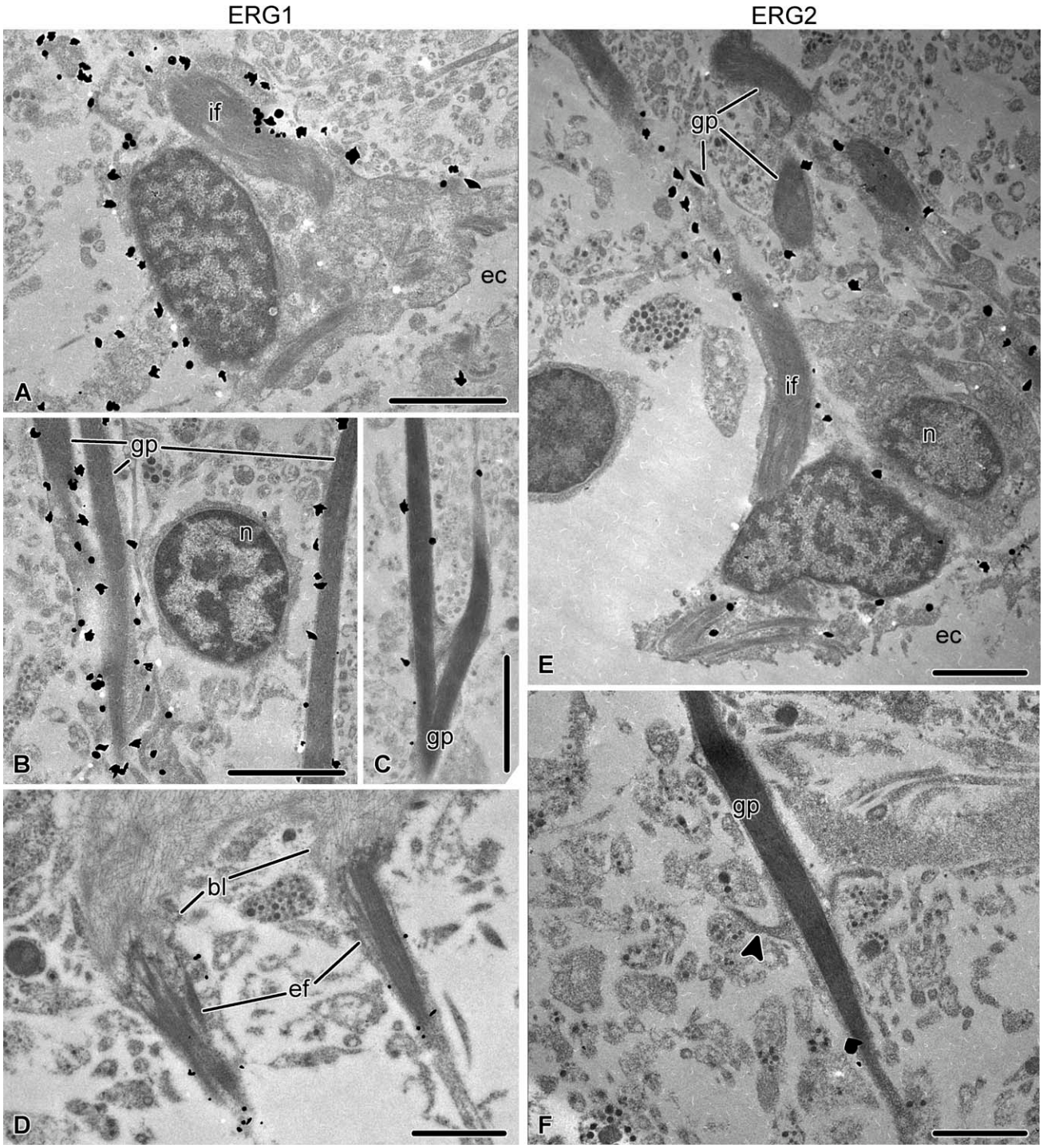

Fig. 4. Immunoelectron microscopy showing the distinct glial characteristics of ERG1-immunopositive cells (**A**–**D**) and ERG2-immunopositive cells (**E**,**F**). (A) ERG1-immmunoreactive glial cell body with characteristic bundles of intermediate filaments (if) in the apical region of the ectoneural neuroepithelium. (B,C) ERG1-positive glial processes (gp) extending through the neural parenchyma and terminating with endfeet (ef) attached to the basal lamina (bl) of the neuroepithelium (D). Note that the processes are occasionally branched (C). (E) Radial glia soma labeled with the ERG2 antibody. (F) The basal processes of glial cells show less pronounced ERG2 immunoreactivity than the cell bodies, which is consistent with immunofluorescence data (see Fig. 3). Note a small lateral protrusion branching off the main process (arrowhead). bl, basal lamina; ec, epineural canal; ef, endfoot; gp, process of glial cell; if, bundle of intermediate filaments; n, neuron. Scale bars: (A–C,E) 2 μm, (D,F) 1 μm.

cucumber. Although an extensive electron microscopy survey suggested that ERG1/ERG2-immunopositive radial glial cells account for the vast majority of non-neuronal cells in the holothurian CNS, to achieve the best possible labeling of glia, we also used the AFRU polyclonal antiserum raised against the bovine Reissner's substance (Rodríguez et al., 1984). As shown earlier, besides labeling the apical regions of the radial glial cells, the

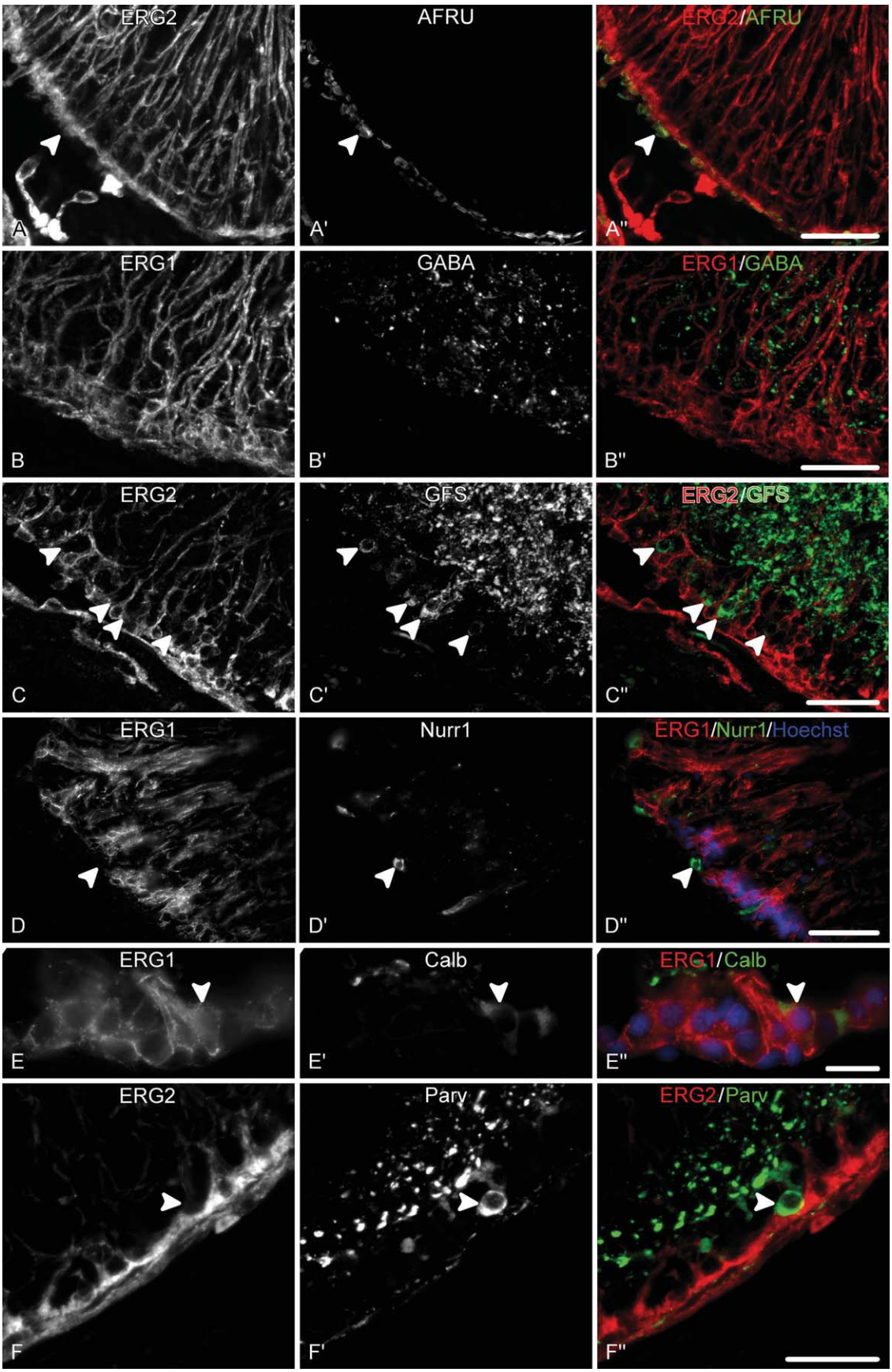

Fig. 5. Representative photomicrographs showing double immunofluorescence labeling of the ectoneural neuroepithelium of the radial nerve cord of *H. glaberrima* with the novel glial markers (ERG1 or ERG2) and other available glia and neuronal markers: **(A–A")** ERG2 and AFRU (a rabbit polyclonal antiserum raised against bovine Reissner's substance; Rodríguez et al., 1984); (**B–B"**) ERG1 and anti-GABA antisera; (**C–C"**) ERG2 and anti-GFSKLYFamide (Diaz-Miranda et al., 1995) antisera; (**D–D"**) ERG1 and anti-Nurr1 antisera; (**E–E"**) ERG1 and anti-calbindin antiserum; (**F–F"**) ERG2 and anti-Parvalbumin antiserum. Arrowheads point to the same areas on images captured in different channels and on corresponding multicolor merged images. A–C" and F–F"' are confocal images (*Z*-projections of image stacks); D–D" and E–E" are epifluorescent micrographs. Scale bars: (A–D") 25 μm, (E–E"') 10 μm, (F–F"') 20 μm.

TABLE 2. Percentage of Immunoreactive Cells (Mean ± SE) in the Central Nervous System of H. glaberrima

RNC region	Cell phenotype		
	ERG1-positive	ERG2-positive	AFRU-positive, but ERG1/ERG2-negative
Radial nerve cord			
As a whole	64.34 ± 2.43	61.79 ± 2.73	1.07 ± 0.28
Ectoneural band	62.47 ± 3.90	59.80 ± 2.19	0.90 ± 0.22
Hyponeural band	68.77 ± 2.65	70.53 ± 4.90	1.62 ± 0.44
Nerve ring	45.06 ± 0.34	43.27 ± 1.30	0.02 ± 0.0001

AFRU antiserum also reveals immunopositive cells bodies scattered in the neural parenchyma. These cells show no labeling with ERG1/ERG2 antibodies and are extremely rare, constituting no more than 2% of the total cell number (Table 2).

Quantitative comparisons (Table 2) did not reveal any significant difference in the relative numbers of the cells recognized by ERG1 and ERG2 in any given region of the nervous system ($P > 0.11$, two-tailed t-test), thus providing statistical support to the morphological data that the two novel antisera labeled the same cell type. Our next analyses investigated whether relative numbers of radial glial cells displayed some pattern of regional variation within the holothurian CNS. The ectoneural and hyponeural bands of the radial nerve cord did not differ significantly from each other in their percentage of glial cells. In both components of the radial nerve, the radial glial cells constituted about 60–70% of the total cell population (Table 2). In contrast, the circumoral nerve ring (which contains only the ectoneural component in sea cucumbers) had significantly smaller relative numbers of glial cells (~45%) than the ectoneural band of the RNC ($P < 0.05$, two tailed t-test).

TUNEL and BrdU Incorporation Assays

TUNEL labeling suggests that, although rare, there are always singly scattered dying cells both in the ectoneural and hyponeural components of the sea cucumber CNS (Fig. 6A). Combined TUNEL labeling and immunofluorescent labeling with the novel antisera shows that both ERG1/ERG2-positive ("glia") and immunonegative cells ("neurons") undergo apoptosis (Fig. 6B–C"). To determine whether the dying cells are replaced through cell division and to characterize the phenotype of dividing cells, we performed a BrdU incorporation assay combined with cell type-specific immunolabeling. Short (24–48 h) exposures to BrdU rarely yielded labeled cells (data not shown); therefore, longer exposure times were used to reliably visualize the BrdU-incorporating cells, which, after 7 days of daily intracoelomic injections of the thymidine analog, comprised 2.71 ± 0.94% (mean ± SE) of the total cell population in the radial nerve cord. Most of the BrdU-positive cells (over 80%) also showed positive immunoreactivity with the novel glia-specific marker (ERG1; Fig. 6D–D", Table 3), whereas only some of the BrdU+ cells were reliably identified as cells exhibiting neuronal phenotype (Fig. 6E–E").

DISCUSSION

Antibody Characterization and Morphological Features of Glial Cells

The ERG1 and ERG2 antibodies label antigens, which, within the nervous system of adult sea cucumbers, are specifically expressed in radial glial cells. These cells have long been recognized as the non-neuronal component of the echinoderm nervous tissue (Bargmann et al., 1962; Mashanov et al., 2006, 2009; Märkel and Röser, 1991; Smith, 1965; Viehweg et al., 1998), but, in spite of repeated attempts to label them with a variety of available antisera, echinoderm glia have never shown expression of "standard" vertebrate glial antigens, such as GFAP, vimentin, glutamine synthetase, and S100. Unfortunately, these markers are of limited utility even for studies on vertebrates because there is currently no single glia-specific antigen that is definitive for glial cell types across the spectrum of taxa (Chanas-Sacre et al., 2000; Howard et al., 2008; Sofroniew and Vinters, 2010). The cell-type specificity of the ERG1 and ERG2 antibodies is supported by two lines of evidence. Immunoelectron microscopy shows that the labeling is exclusively associated with the cells of the nervous tissue, whose ultrastructural features indicate their non-neuronal nature. Moreover, double immunofluorescence labeling assays show no overlap between ERG1 or ERG2 and neuronal markers. The only exception is calbindin-d28k-like immunoreactivity of some of the radial glial cells. Calbindin is a member of calcium-binding protein superfamily implicated in the regulation the intracellular Ca^{2+} level. Although calcium-binding proteins were originally thought to be specifically expressed in certain populations of nerve cells in the central and peripheral nervous system of vertebrates (Andressen et al., 1993), calbindin(-like) immunoreactivity has been reported in cells of glial nature, such as ependymocytes, radial glial cells, and reactive astrocytes (Liu and Graybiel, 1992; Manso et al., 1997; Mattson et al., 1995). Our immunocytochemical assays show calbindin-like immunoreactivity only in some of the radial glial cells, thereby suggesting that the radial glia are not homogeneous, falling at least into calbindin-positive and calbidin-negative subpopulations. The physiological function of calbindin expression in the radial glia is not fully understood. However, in this regard, it is interesting to mention a recent discovery of spontaneous calcium waves, which propagate through the radial glial cells of the developing mammalian brain and are thought to modulate cell proliferation during cortical development (Weissmann et al., 2004).

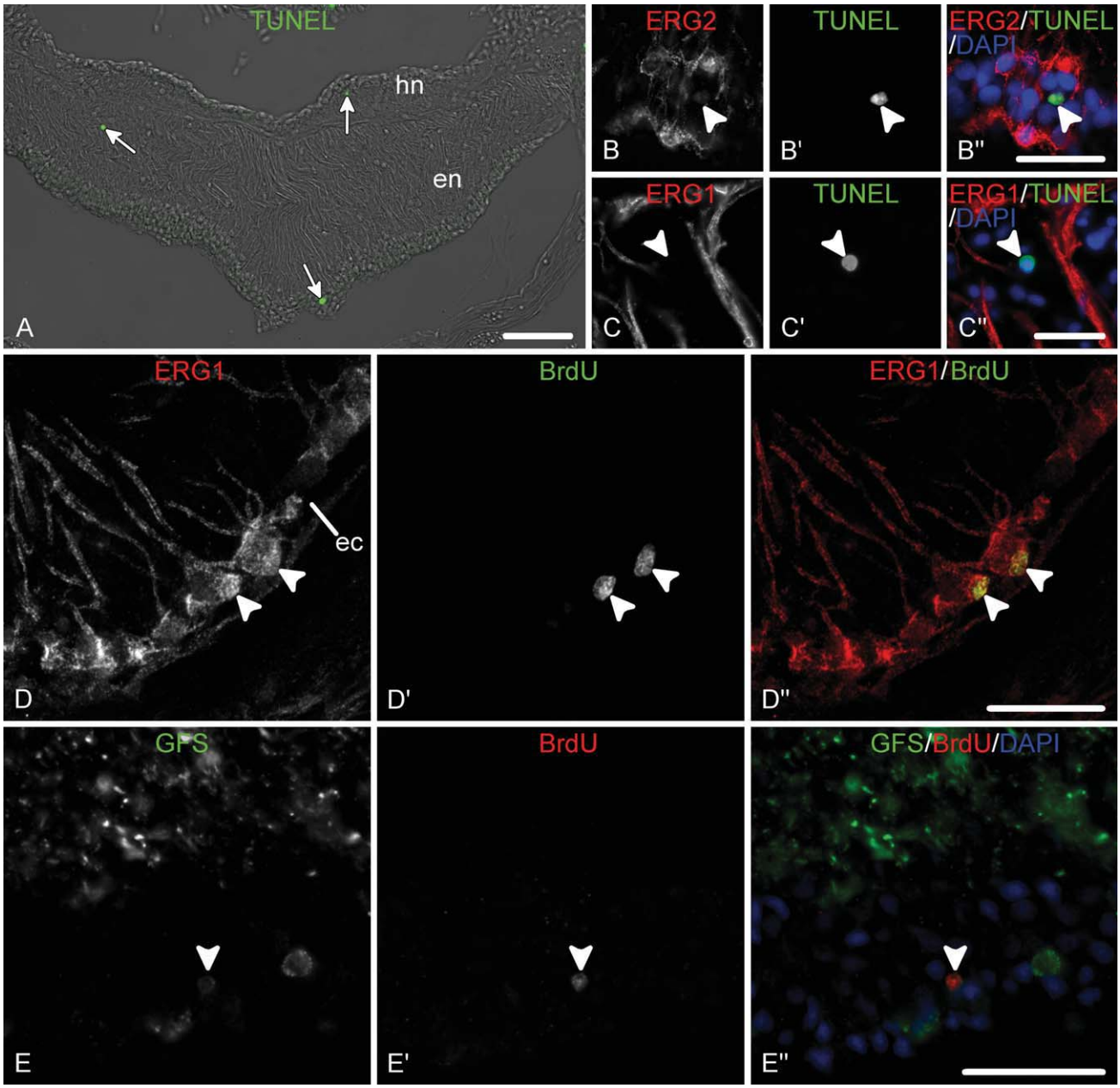

Fig. 6. Apoptosis (**A–C"**) and cell division (**D–E"**) in the adult sea cucumber nervous system. (A) TUNEL assay, low-magnification overview of a cross section of the radial nerve cord. (B–B") Double staining for TUNEL and ERG2, showing a dying ERG2-immunopositive cell. (C–C") Double labeling for TUNEL and ERG1; note a dying ERG1-negative cell ("neuron"). (D–E") Representative micrographs of BrdU cell proliferation assays. (D–D") Most of the BrdU-positive cells were also ERG1/ERG2-immunopositive. Only few of BrdU-positive cells show neuronal immunophenotype (for instance, positive immunolabeling with the anti-GFSKLYFamide antiserum) (E–E"). In B–E", arrowheads point to the same areas of images taken in different channels and on resulting composite images. ec, epineural canal; en, ectoneural part of the radial nerve cord; hn, hyponeural part of the radial nerve cord. Scale bars: (A) 50 μm, (B–E") 20 μm.

Therefore, although the nature of the antigens is not yet known, the robustness and specificity of labeling and fine level of details that the staining provides make the novel ERG1 and ERG2 antibodies a valuable tool to study the echinoderm glia under normal physiological conditions and also in various experimental settings, including post-traumatic regeneration.

The morphological and immunocytochemical features of echinoderm radial glial cells (see Fig. 7) not only clearly set them apart from neurons but also share a great deal of similarity with the characteristics of the radial glia of chordates, a ubiquitous cell type in the developing CNS, which also persists into adult in lower vertebrates (Chanas-Sacre et al., 2000; Götz et al., 2002). The key features in common include the elongated shape of the cells with processes extending through the neural parenchyma, their orthogonal orientation relative to the surfaces of the neuroepithelium,

TABLE 3. Percentage (Mean ± SE) of BrdU Incorporating Cells in the Radial Nerve Cord of H. glaberrima After Daily Injections for 7 Days

Type of percentage	Ectoneural part of the RNC	Hyponeural part of the RNC	RNC, total
Number of BrdU+ cells relative to the total number of cells	2.74 ± 0.87	2.51 ± 1.23	2.71 ± 0.94
Number of ERG1+ BrdU+ cells relative to the total number of BrdU+ cells	86.19 ± 4.13	84.13 ± 9.66	85.97 ± 3.14

conspicuous well-organized bundles of intermediate filaments, short side branches (termed lamellate expansions in mammals) protruding from the main process(es), and ability to produce and secrete so-called Reissner's substance. This suggests that the fundamental histological architecture of the centralized nervous system in deuterostomes, with the radial glia forming a dense supporting framework of vertical fibers and neuronal somata and processes filling in the space between them, is a phylogenetically ancient trait, which could have predated the diversification of Chordata and Ambulacraria.

Glial Cell Quantification

Since glial cells are known to be involved in multiple functional relationships with neurons (Allen and Barres, 2009; Sofroniew and Vinters, 2010), the relative number of glial cells, or glia/neuron ratio, has been considered of great relevance and was used as an indicator of various crucial aspects of the nervous tissue biology. Glia/neuron ratio is thought to be dependent on the size of the CNS (Allen and Barres, 2009; Hawkins and Olszewski, 1957; Reichenbach, 1989), metabolic demand of neighboring neurons (Sherwood et al., 2006), and age (Pelvig et al., 2008), and it can also be affected by pathological conditions (Ongür et al., 1998) and environmental factors (Szeligo and Leblond, 1977).

In our quantitative studies, we applied the ERG1 and ERG2 monoclonal antibodies and the AFRU polyclonal antiserum to visualize glial cells. The latter antiserum was raised against bovine Reissner's substance, the material produced by secretory radial glia of the subcomissural organ in chordates (Rodríguez et al., 1984). In the holothurian nervous system, this antibody labels the apical regions of the ERG1/ERG2-immunopositive radial glial cells, but also rare (about 2% of the total cell number) cells in the neural parenchyma, which are not labeled with ERG1 and ERG2. For the purposes of this study, we can determine the total size of the glial subpopulation by summing up the numbers of the ERG1/ERG2-immunopositive radial glia and the parenchymal AFRU-immunoreactive cells. Our estimates provide the glia/neuron ratios of about 1.89 (when ERG1 was used to label the radial glia) or 1.69 (when the radial glia was labeled with ERG2) for the radial nerve cord and 0.82 or 0.76 (for ERG1 and ERG2 labeling of the radial glia, respectively) for the circumoral nerve ring of *H. glaberrima.* Surprisingly, these numbers do not differ much from the range of glia/neuron ratio of 0.32–3.76 reported for different parts of the human brain (Azevedo et al., 2009). Therefore, the simple rule that larger nervous systems have greater glia/neuron ratio (Hawkins and Olzewski, 1957; Reichenbach, 1989), although being true, for some groups of mammals (Herculano-Houzel et al., 2006) does not necessarily apply at a larger phylogenetic scale. Moreover, the above comparison also suggests that even relatively simple centralized nervous systems, like the echinoderm CNS, seem to require a certain minimal number of glial cells per neuron to function properly.

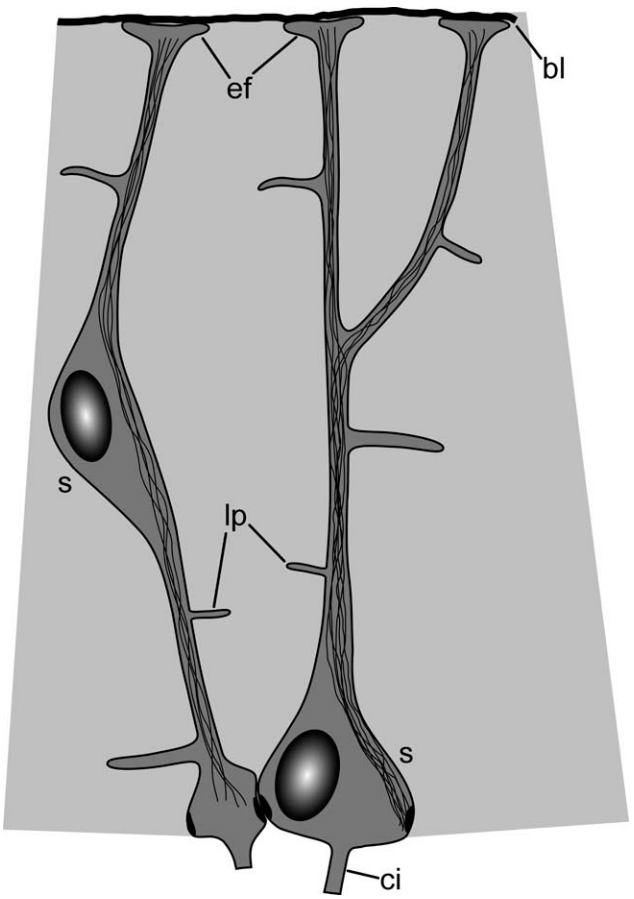

Fig. 7. Diagram summarizing morphological features of radial glial cells in the sea cucumber central nervous system. This cell type is characterized by elongated shape, which allows the cell to span the entire height of the neuroepithelium between the apical and basal surfaces. At their basal end, the glial cells form endfeet (ef), which anchor to the basal lamina (bl) by hemidesmosomes, while their apical surface bears a cilium (ci) protruding into the lumen of the epineural or hyponeural canal (Mashanov et al., 2006).Two morphological variations of glia can be recognized by the position of their somata (s). Unipolar cells have their apical cell body at the surface of the neuroepithelium and a single basal process. The somata of bipolar radial glia are submerged into the neural parenchyma; two processes run in the opposite directions to reach the apical and basal surfaces of the neuroepithelium. The glial processes contain bundles of orderly oriented densely packed intermediate filaments; they occasionally bifurcate and bear short lateral protrusions (lp). Figure not to scale.

Another interesting observation is that there is no statistical difference in the relative numbers of radial glial cells between the ectoneural and hyponeural parts of the radial nerve cord, but there is a significant difference in glia/neuron ratio between the ectoneural nerve ring and the ectoneural band of the RNC. The difference in cell type composition between different parts of the CNS is common. For instance, the glia/neuron ratio of the human brain as a whole is close to 1, but it ranges in specific brain regions structures from 0.23 (cerebellum) to 11.35 (basal ganglia, diencephalon, and the brainstem; Azevedo et al., 2009). The difference in this ratio between the nerve ring and the radial cord in the sea cucumber could reflect a certain degree of regional differentiation, which is present in the seemingly simple CNS of echinoderms. This difference can in turn imply some functional subdivision between the circumoral nerve ring and the radial nerve cord in echinoderms. Although it is generally agreed that the individual radial nerve cords can independently control the adjacent regions of the body, the mechanisms of the coordinated control over the body as a whole remain unknown. There is a considerable disagreement in the literature about the physiological role of the nerve ring. Some authors view the nerve ring as a mere linking system, which connects together the radial nerve cords, visceral and tentacular (in sea cucumbers) nerves (Cobb, 1987), while others suggest that coordinated organismal activities are only possible because the nerve ring houses a set of controlling centers (Smith, 1965). Obviously, further studies are needed to fully resolve this dilemma. Our results suggest that although the basic neurohistological architecture is the same in the nerve ring and the radial nerve cords (Mashanov et al., 2006), the nerve ring contains more neuronal perikarya per 100 cells (about 55%) than the radial nerve cords (about 35%; this study), which, in our opinion, is not consistent with the idea that the only function of the nerve ring is just to provide connection between the radial nerve cords.

Involvement of Glial Cells in Cell Turnover

BrdU immunoreactivity suggests that the labeled cells are engaged in DNA synthesis, which most probably reflects replication in the S-phase of the cell cycle. Therefore, new cells obviously continue to be produced in the neuroepithelia of the adult sea cucumber CNS under normal physiological conditions. Interestingly, most of the BrdU-immunopositive cells are also labeled with the ERG1 and ERG2 antibodies, which specifically stain the radial glial cells. Thus, the radial glia constitute the main proliferating cell population and, therefore, seem to play a major role in the production of new cells in the echinoderm CNS. Interestingly, glial cells of sea cucumbers are capable of differentiating into neurons during post-traumatic regeneration of the radial nerve cord, as evidenced by detailed electron microscopy examinations (Mashanov et al., 2008) and by co-expression of both glial and neuronal markers (Mashanov et al., unpublished). Although it still remains unclear whether echinoderm radial glia can give rise to neurons under normal physiological conditions, our observations are in accordance with the data on cell division in the adult nervous systems of chordates and arthropods, where stem/progenitor cells in adult neurogenesis have been shown to display glial characteristics (Basak and Taylor, 2009; Kriegstein and Alvarez-Buylla, 2009; Merkle et al., 2004; Sullivan et al., 2007). Our data add up to the emerging generalization that the plasticity in the adult CNS, including the ability to produce new cells as well as post-traumatic regeneration, depends on the maintenance of the radial glia characteristics into adulthood (Götz et al., 2002; Tanaka and Ferretti, 2009).

Apoptosis, as a form of programmed cell death, has an important role in normal tissue turnover, post-traumatic regeneration, embryonic development, and metamorphosis. In mammalian brain development, the programmed cell death is a mechanism, which controls the final size and shape of the brain and allows rapid elimination of neurons that failed to establish proper synaptic connections (Oppenheim, 1991). In the adult brain, cell death has been frequently seen within the neurogenic zones, where both excess immature and some fully differentiated cells are eliminated (Kuhn, 2008). In our experiments, apoptosis is regularly detected in the adult CNS of the sea cucumber. Co-labeling assays suggest that both the radial glial cells (ERG1- or ERG2-immunopositive) and neurons (the cells that are not labeled with ERG1 and ERG2) undergo some degree of cell death, although the functional significance of it remains unclear.

CONCLUSION: PHYLOGENETIC IMPLICATIONS

The CNS of the sea cucumber *H. glaberrima* possesses an abundant non-neuronal cell type, which shows deep similarities with the radial glia in chordates. The basic common features include (a) an elongated shape, (b) long radially oriented processes, forming a dense network of fibers, (c) short lateral protrusions branching off the main processes, (d) prominent bundles of intermediate filaments, and (e) ability to produce Reissner's substance. Radial glial cells of both echinoderms and chordates play a crucial role in producing new cells in the adult nervous system. These similarities suggest a possible homology between the radial glial cells in both phyla. Therefore, radial glia could be an ancestral feature of the deuterostome CNS, and the origin of this cell type might have predated the diversification of the Chordata and Ambulacraria (Echinodermata + Hemichordata) lineages.

However, along with the similarities, there are important differences between echinoderms and chordates in organization of glial cell population. In mammals, radial glia predominate during embryonic development, but eventually largely disappear giving rise both to neurons and the extraordinary diversity of specialized glial cell types, including astrocytes, ependymocytes, oligodendro-

cytes, and also stem/progenitor cells in restricted germinal regions of the adult CNS (Kriegstein and Alvarez-Buylla, 2009; Malatesta et al., 2008; Spassky et al., 2005). In lower vertebrates, radial glia persist into adulthood, but co-exist with other abundant glial cells, such as oligodendrocytes and ependymocytes (Tanaka and Ferretti, 2009; Zamora, 1978). Moreover, a surprisingly high level of glial diversity has been reported even in amphioxus, a basal member of the chordate clade, whose nerve cord, besides radial glial cells, contains at least three other non-neuronal cell types (Lacalli and Kelly, 2002). In the CNS of *H. glaberrima*, we have not seen analogs of astrocytes, oligodendroglia, or microglia. Besides very rare Reissner's substance-immunopositive cells in the neural parenchyma (Mashanov et al., 2009; this study), the entire glial population in the echinoderm neuroepithelia is represented by radial glia. Therefore, the nervous system architecture in echinoderms, with the radial glia being the only major non-neuronal cell type, can be the basal condition within the Deuterostomia, which might have been in place already at the early stages of deuterostome evolution, as were, for instance, the basic molecular mechanisms underlying the CNS patterning (Lowe, 2003).

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