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REGULAR ARTICLE

Immunocytochemical localization of a putative strychnine-sensitive glycine receptor in *Hydra vulgaris*

Linda A. Hufnagel[1] · Paola Pierobon[2] · Gabriele Kass-Simon[3]

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Abstract

Previous biochemical studies have identified strychnine-sensitive glycine receptors in membrane preparations of *Hydra vulgaris* (Cnidaria: Hydrozoa). Electrophysiological and behavioral evidence has shown that these receptors play a role in modulating pacemaker activity and feeding behavior. Here, we present our genomic analysis that revealed hydra proteins having strong homology with the strychnine-binding region of the human receptor protein, GlyRα1. We further present immunocytochemical evidence for the specific labeling of cell and tissue preparations of hydra by a commercially available polyclonal anti-GlyRα1 antibody, selected through our genomic analysis. Tissue pieces and cell macerates from the upper and lower thirds of the body and ablated tentacles were double-labeled with this antibody and with an antibody specific for α-tubulin, to identify the glycine receptors and microtubules, respectively. Extensive receptor labeling was evident on the membranes, cell bodies and myonemes of endodermal and ectodermal epithelial cells, cell bodies and neurites of nerve cells, cnidocytes and interstitial cells. Labeling of the membranes of epithelial cells frequently corresponded to conspicuous varicosities (presumptive presynaptic sites) in the associated nerve net. Our findings support the idea that glycine receptors form an integral part of the nerve and effector systems that control hydra behavior.

Keywords Amino acid transmitters · GABA · Cnidocytes · Nerve net · Interstitial cells

Introduction

The amino acid neurotransmitter glycine, long identified as the inhibitory transmitter of the Renshaw cells of the vertebrate spinal cord (Curtis et al. 1976), has increasingly gained importance as an inhibitory transmitter of the vertebrate brain (Baer et al. 2009). The recognition of glycine's role in vertebrates has raised the question of its existence as an inhibitory transmitter in the invertebrates (Laughton et al. 1994, 1995; Pierobon et al. 2001; Ruggieri et al. 2004; Pierobon 2015) and its place on the metazoan evolutionary tree.

By binding to its receptor, glycine directly produces an inward, chloride current that hyperpolarizes a given cell. The receptor is characterized by the distinct pharmacology of its ligands. The chloride current through the receptor is elicited by β-alanine and taurine, in addition to glycine (Langosch et al. 1990) and is blocked by strychnine (Young and Snyder 1973, 1974). The glycine receptor (GlyR) belongs to the Cys-loop family of the ligand-gated ion channel superfamily (LGIC), which includes the $GABA_A$ receptor, the $5HT_3$ receptor and the nicotinic acetylcholine receptor (Ortells and Lunt 1995). It is a heteromeric pentameric protein complex composed of 48 kDa alpha subunits, on which the binding sites for

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✉ Linda A. Hufnagel
lhufnagel@uri.edu

✉ Gabriele Kass-Simon
gkass-simon@uri.edu

Paola Pierobon
p.pierobon@isasi.cnr.it

[1] Department of Cell and Molecular Biology & Interdisciplinary Neurosciences Program, University of Rhode Island, Flagg Road, Kingston, RI 02881, USA

[2] Institute of Applied Sciences and Intelligent Systems E. Caianiello, CNR, Via Campi Flegrei 34, 80078, Pozzuoli Naples, Italy

[3] Department of Biological Sciences & Interdisciplinary Neurosciences Program, University of Rhode Island, Flagg Road, Kingston, RI 02881, USA

Fig. 1 CLUSTAL Omega multiple sequence alignment of hydra protein #1 with the strychnine-binding region (AA187-231) and the immunogen of the human GlyRα1 (AA 178-199). The amino acid sequences of the strychnine-binding region and the immunogen are essentially coincident (between E1 of the strychnine-binding region and T50 of the immunogen), except for a short upstream sequence on the immunogen and a short downstream sequence on the strychnine-binding region. Between S178 and N212 in the hydra protein, there are several regions of strong homology. Note that at the C-terminal of the human strychnine-binding region, there are only four amino acids (KHYN) that are not found in the immunogen sequence. Note also the shared presence of the GY sequence (Gly160, Tyr161 in the human protein in the literature and Gly180, Tyr181 in the hydra protein in the figure) requisite in the human protein for strychnine beginning of a five amino acid region of very strong homology (SYGYT in the hydra protein, overlined). * = identity,: = strong functional similarity, . = functional similarity

glycine and strychnine are located and 58 kDa beta subunits (Betz et al. 1999; Lynch 2004; Dutertre et al. 2012).

The pentameric ligand-gated ion channels (LGIC) constitute a large family of membrane proteins that are expressed in animals and certain bacteria (Corringer et al. 2012). A growing number of studies of invertebrate LGICs show a surprisingly high degree of conservation in the structure of receptor subunits (Tasneem et al. 2004; Liebeskind et al. 2015). Their molecular architecture and function are conserved throughout the family. In mammals, they operate as receptors of the neurotransmitters acetylcholine, serotonin, GABA and glycine and play a key role in electrical signal transduction at chemical synapses. Invertebrate LGICs also include the inhibitory glutamate-gated chloride channel family (Cockroft et al. 1990; Cleland 1996).

Our previous biochemical, behavioral and physiological studies on the early evolved metazoan *Hydra vulgaris* have provided evidence that a strychnine-sensitive glycine

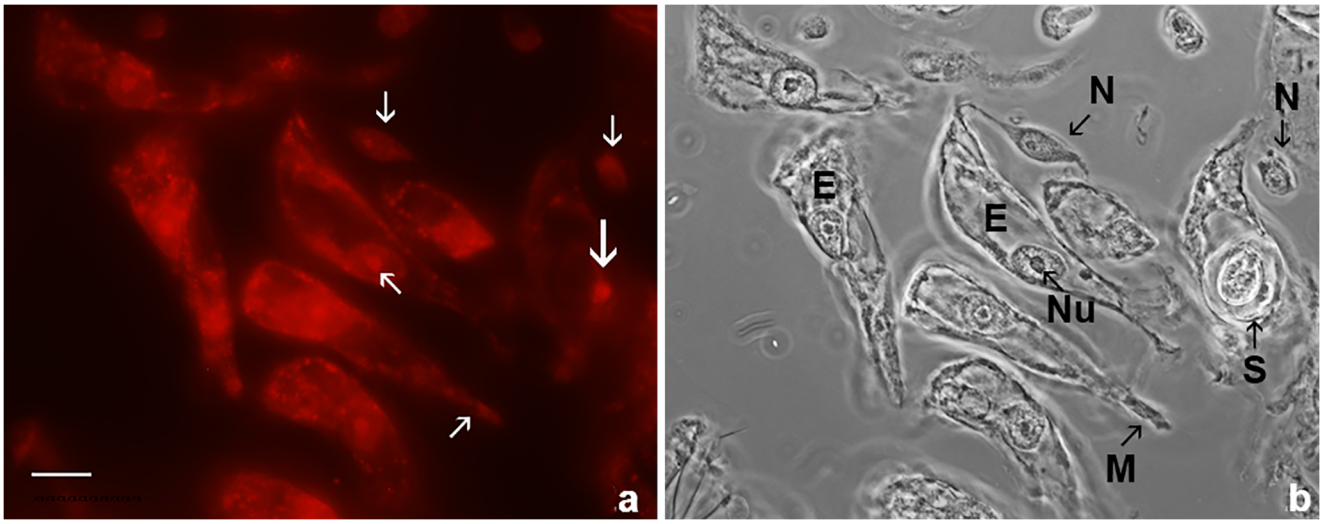

Fig. 2 Endodermal EMCs (E) from the head region, with associated stenotele cnidocyte (S) and developing nerve cells (N). Note patches of anti-glyR labeling on a myoneme (M) and lateral and apical surfaces of the EMCs, uniformly distributed labeling within the cytoplasm of EMCs and developing nerve cells, and dense uniformly distributed labeling of nucleoli (Nu) of EMCs (small arrows). A bright region of label is present adjacent to the capsule of the stenotele cnidocyte (large arrow). **a** Texas Red, **b** phase contrast. Bar = 10 μm

receptor exists in its cell membranes. The receptor has been shown to be involved in prolonging the duration of the mouth opening component of its feeding response, by inhibiting mouth closure (Pierobon et al. 2001). In electrophysiological experiments, we presented evidence that the receptor is involved in inhibiting the output of hydra's primary pacemaker systems, reducing the impulse frequency of its rhythmic potential (RP) system and the output of hydra's body contraction burst (CB) system (Ruggieri et al. 2004). Recent findings indicate that it has an inhibitory role in hydra's mouth-opening behavior (Pierobon 2015).

Apart from its inhibitory function through the strychnine-sensitive receptor, glycine also plays a role in

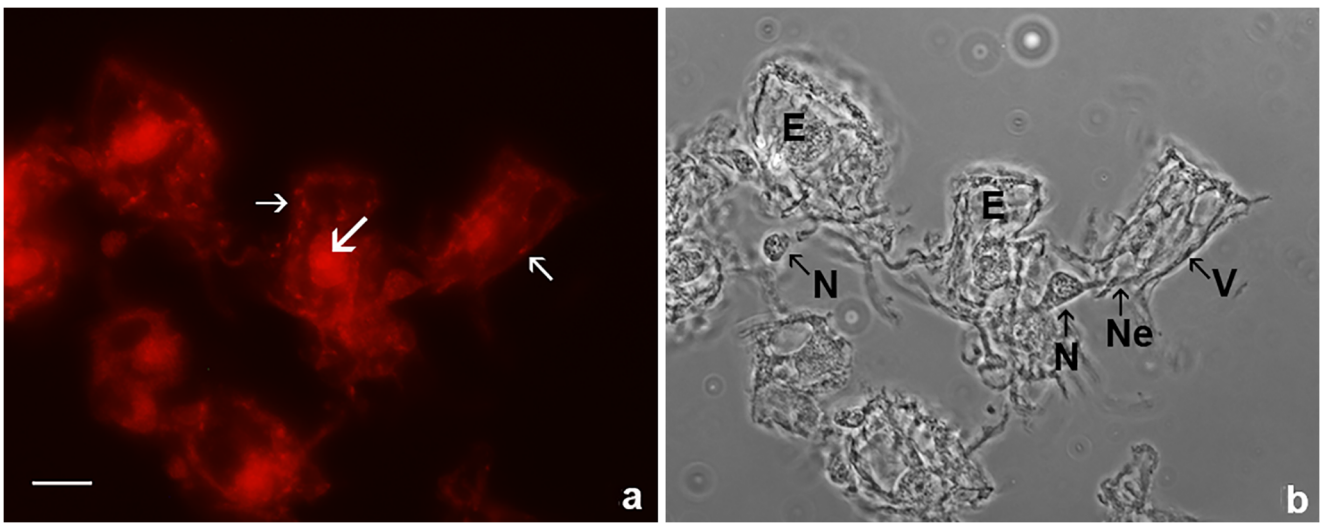

Fig. 3 Ectodermal EMCs (E) and nerve cells (N) and portions of the ectodermal nerve net from the lower body region. Note patches of anti-glyR labeling on the lateral surfaces of the EMCs (small arrows), intense labeling of a nucleolus (large arrow), and moderate labeling of the EMC nucleoplasm. The cytoplasm and nucleoplasm of nerve cells are moderately labeled. Neurites (Ne) appear to be part of a network associated with one of the EMCs, which follows along its borders. Its varicosities (V) appear to be associated with labeled patches (a, small arrows). **a** Texas Red, **b** phase contrast. Bar = 10 μm

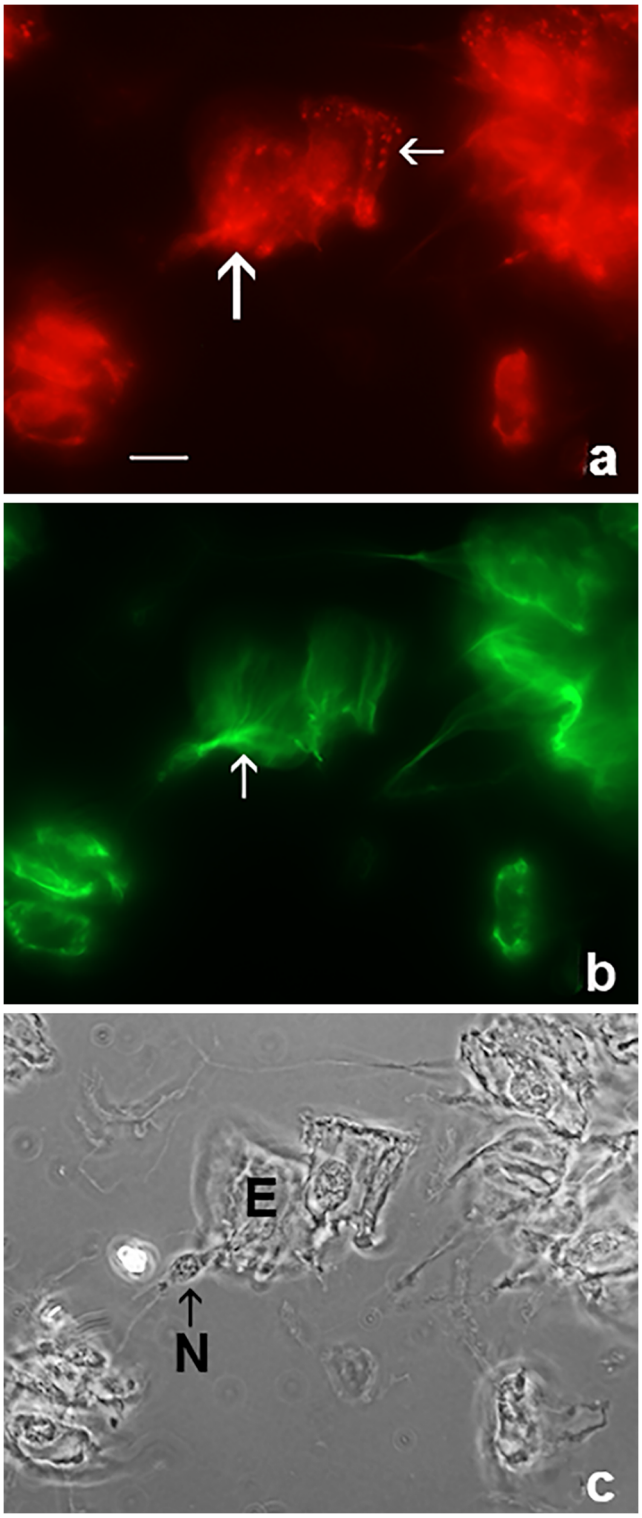

Fig. 4 Ectodermal EMCs (E) with an associated nerve cell (N) from the lower body region. Note the rows of small, regularly spaced patches of anti-glyR label aligned with folds of an EMC (a, small arrow). Microtubules, revealed by anti-tubulin labeling, run in parallel with the rows of patchy anti-glyR labeling (b). Associated with another EMC is a prominent neuron (N) whose neurites splay out over the cell body of the EMC (b, arrow). Abundant anti-glyR labeling is associated with the region of neurite branching (a, large arrow). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

cellular excitation. Glycine is required for activation of the excitatory glutamate NMDA receptor (Kleckner and Dingledine 1988). On the NR1 subunit of the NMDA receptor is a strychnine-insensitive glycine site (Danysz and Parsons 1998), whose agonists and antagonists include D-serine (Mothet et al. 2000) and indole-2-carboxylic acid (Huettner 1989). A corresponding role for glycine as an NMDA co-agonist has been found in hydra, where it affects feeding behavior (Pierobon et al. 2004). Its putative agonist, D-serine, contributes to the effect of NMDA on hydra's tentacle pacemaker systems (Kay and Kass-Simon 2009) and cnidocyst discharge (Kass-Simon and Scappaticci 2004; Scappaticci and Kass-Simon 2008); NMDA receptors have been localized in hydra's nerve and effector cells (Scappaticci et al. 2004). Thus, glycine can bind to both excitatory glutamatergic NMDA receptors and inhibitory strychnine-sensitive receptors in hydra and appears to affect hydra's behavior through both of these receptors.

In order to delineate the morphological underpinnings of glycine's inhibitory and excitatory roles in hydra, we undertook the localization of the strychnine-sensitive glycine receptor by means of antibodies specifically targeted to the strychnine-binding site of the glycine receptor. The appropriateness of the antibody used for the identification of glycine receptors was established by extensive genomic analysis.

Materials and methods

Culturing methods All experiments were carried out on *Hydra vulgaris*, maintained at 18 ± 1 °C in glass culture dishes in a modified M solution (Muscatine and Lenhoff 1965), containing $NaHCO_3$ (1×10^{-3} M), $CaCl_2$ (2.5×10^{-4} M), $MgCl_2$ (5×10^{-4} M) and EDTA (1×10^{-5} M) in distilled water. Animals were fed with *Artemia* nauplii on alternate days; culture solution was changed 1 h after feeding. Sample animals were randomly chosen from groups of polyps starved 4 to 5 days prior to the experiment.

Immunocytochemical methods For immunocytochemistry, ablated heads (tentacles and hypostome), gastric regions, or peduncles were placed on agar-coated glass slides, macerated for about 5 min in a dissociation medium (glycerol:acetic acid:deionized water, 1:1:7) (Scappaticci et al. 2004) and immediately fixed with Lavdovsky's fixative (Dunne et al. 1985) for 1 h. After washes with 10 mM phosphate-buffered saline (PBS) and 10 mM modified phosphate-buffered saline, MPBS/BSA (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.02% KCl, 0.1% polyethylene glycol 20, 0.2% Tween 20, 0.05% Na azide, 1% bovine serum albumin fr. V), the slides

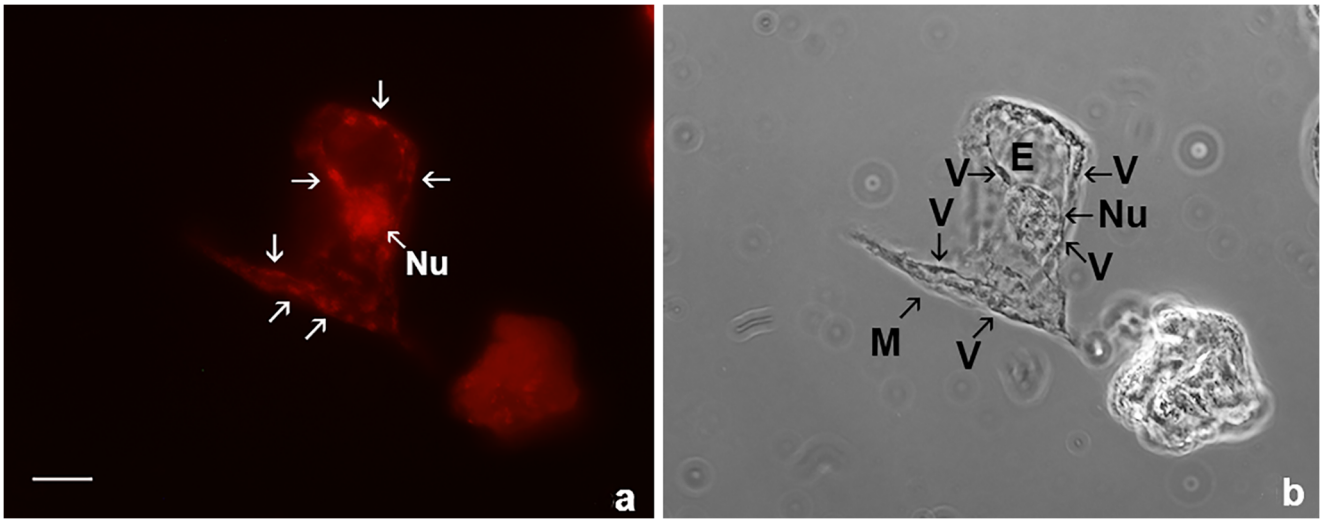

Fig. 5 Ectodermal EMC (E) from the lower body region. Patches of bright labeling are evident on the myoneme (M) and lateral and apical surfaces of the cell body (a, arrows). The patches of labeling appear coincident with dense varicosities (V) along nerve processes that parallel the myoneme and run diagonally up the surface of the cell body. There is diffuse labeling within the nucleoplasm (Nu) and brighter labeling of the enclosed nucleolus. **a** Texas Red, **b** phase contrast. Bar = 10 μm

were incubated overnight with a 1:400 dilution of primary antibody, rabbit polyclonal anti-glycine receptor antibody (anti-GlyR1; abcam ab26134), raised against residues 178–227 of the human GlyRα1 subunit. This antibody was selected from several commercially available antibodies because of the correspondence of a large portion of its immunogen sequence with all but four amino acids of the strychnine-binding site of the human glycine receptor (see below) and because of the strong homology between its immunogen and a putative hydra glycine receptor sequence that was identified through sequence alignment methods (Fig. 1). Macerates were then washed and treated with a 1:400 dilution of secondary antibody, AlexaFluor 488– or Texas Red–labeled goat anti-rabbit IgG (H + L) (InVitrogen/Molecular Probes T2767). In double-labeling experiments, a primary antibody mixture containing the above-described anti-GlyRα1 antibody with a mouse monoclonal anti-α-tubulin antibody, Sigma clone B512 (T/5168) (1:1000 dilution) was used. This was followed by a secondary antibody mixture containing AlexaFluor 488-labeled goat anti-mouse IgG (H + L) (InVitrogen/Molecular Probes A11029) (1:400 dilution) and Texas Red–labeled anti-rabbit IgG as described above. The macerates were covered with antifade mounting medium (Prolong Gold, Molecular Probes) and coverslips were applied. The slides were examined and digital photographs were taken with a Zeiss AxioPlan 1 imagining system equipped with epifluorescence filters and a Zeiss AxioCam color digital camera.

Negative control slides, in which primary antibody was omitted, were included in all experiments. Specific binding of primary antibodies was demonstrated by the fact that negative control slides revealed only very low-level general background fluorescence, which was not detectable under the imaging conditions used for photographing the experimental slides.

Identification of cell types Cells were identified visually by their resemblance to those previously described and identified in our and other workers' publications (Hadzi 1909; David 1973; Hufnagel et al. 1985)

Genomic analysis We performed a series of genomic database analyses using the BLAST function at NCBI. Blasts were carried out against *H. vulgaris*– or *H. sapiens*–predicted protein sequences in the NCBI web-accessible protein database, as follows:

1) The entire human GlyRα1 sequence (AAH74980.1) against all *H. vulgaris* protein sequences, producing an output of 26 proteins with e-values of $5e^{-10}$ or lower. These were numbered 1 through 26.
2) The amino acid sequence (AA178-227) of the immunogen used to produce the antibody employed in our study against all predicted *H. vulgaris* protein sequences.
3) The human GlyRα1 strychnine-binding site sequence (AA187-231) against all *H. vulgaris*–predicted protein sequences.

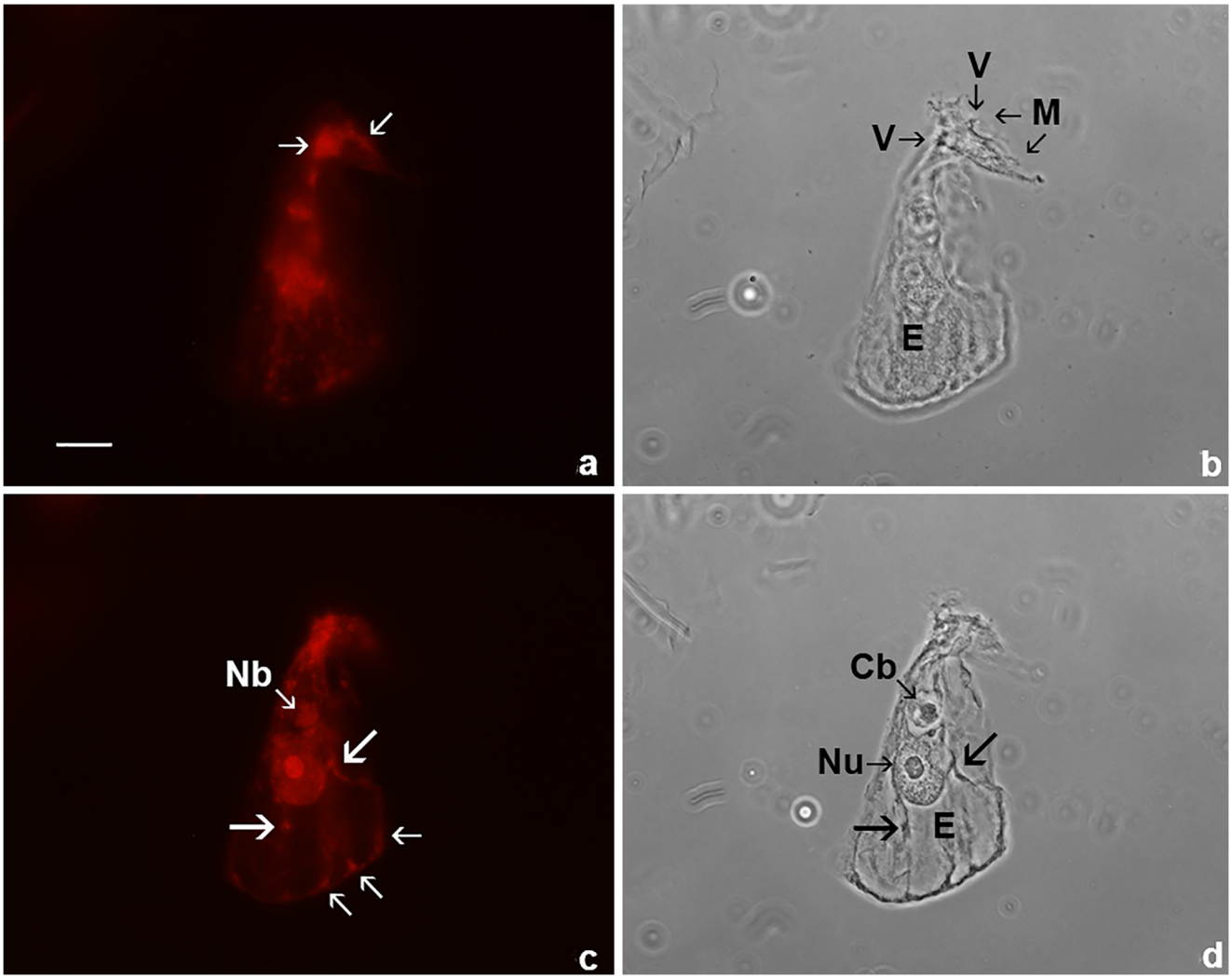

Fig. 6 Endodermal EMC (E) from the lower body region; two focal planes (a, b and c, d). The EMC myoneme (M), and varicosities (V) of the associated nerve net are evident (b). Patches of bright anti-glyR labeling can be seen on the myoneme and lateral and apical surfaces of the cell body (a, c, small arrows). There is diffuse labeling within the nucleoplasm (Nu) and brighter labeling of the enclosed nucleolus (c, d). Patches of labeling appear coincident with dense varicosities (V) of neurites that run diagonally up the surface of the cell body (c, d, large arrows). A putative developing cnidoblast (Cb) lies in a pocket on the side of the cell (d). **a**, **c** Texas Red; **b**, **d** phase contrast. Bar = 10 μm

4) The *H. vulgaris* protein sequence (XP_012560050), designated as #1 in our blast using the entire human GlyRα1 protein sequence against all predicted human protein sequences.

In addition, we performed blast alignments:

1) The *H. sapiens* GlyRα1 sequence with four *H. vulgaris* protein sequences (#1; #2: XP_012560049; #9: XP_012556838; #18: XP_012555765).
2) The human GlyRα1 strychnine-binding site sequence with selected *H. vulgaris* protein sequences.

We performed pairwise sequence alignments using EMBOSS Matcher and EMBOSS Needle (http://www.ebi.ac.uk/) and multiple sequence alignments using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to compare sequences of interest.

Results

Genomic analysis

A region of strong homology was found among several hydra proteins, the immunogen and the strychnine-binding site of the human GlyRα1 subunit based on the following analyses.

We blasted the entire *H. vulgaris* protein database with the whole human GlyRα1 protein sequence. Twenty-six proteins with e-values better than e^{-10} were recognized and assigned

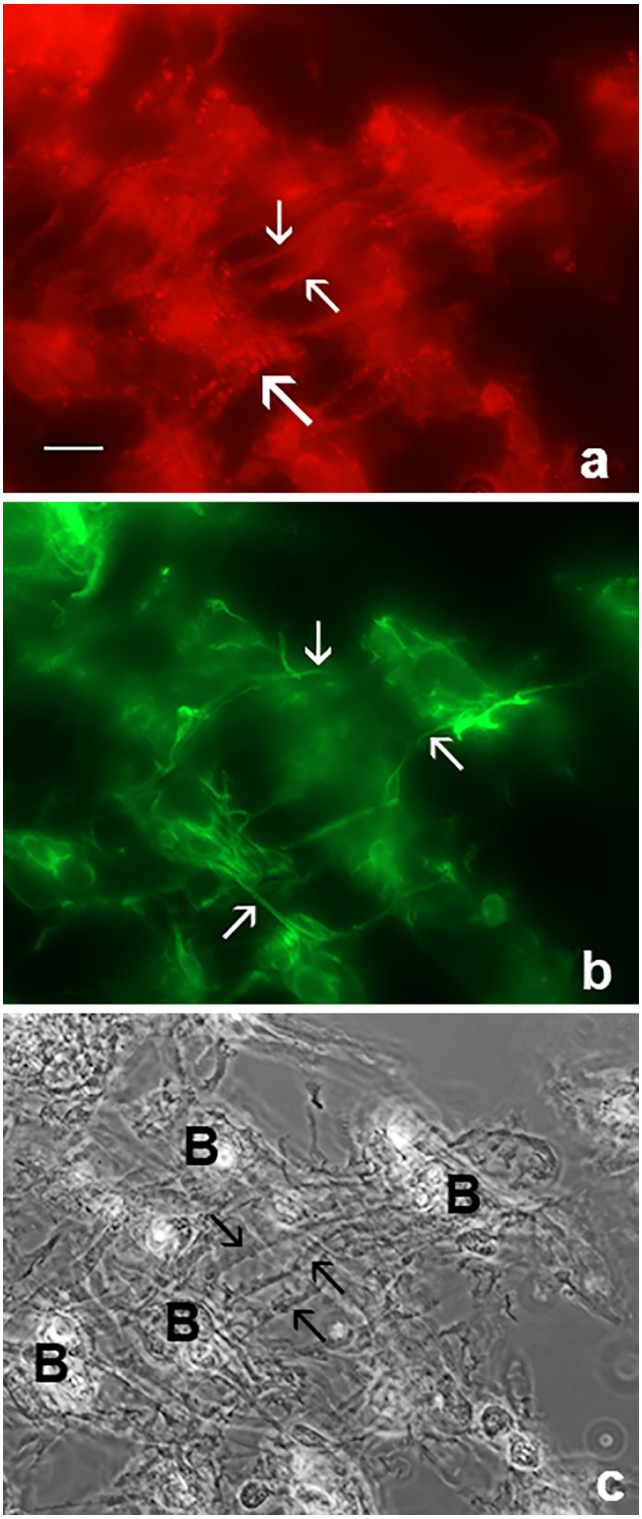

Fig. 7 Battery cell complexes (B) of the tentacle connected by their myonemes (c. arrows) and neurites (7b, arrows). Note abundant patches of anti-glyR label on the myonemes (a, small arrows), particularly in the vicinity of the battery cell bodies. Also note intense anti-glyR labeling within the interior of the battery cell complexes. Patchy label is evident where neurites and myonemes converge (a, large arrow). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

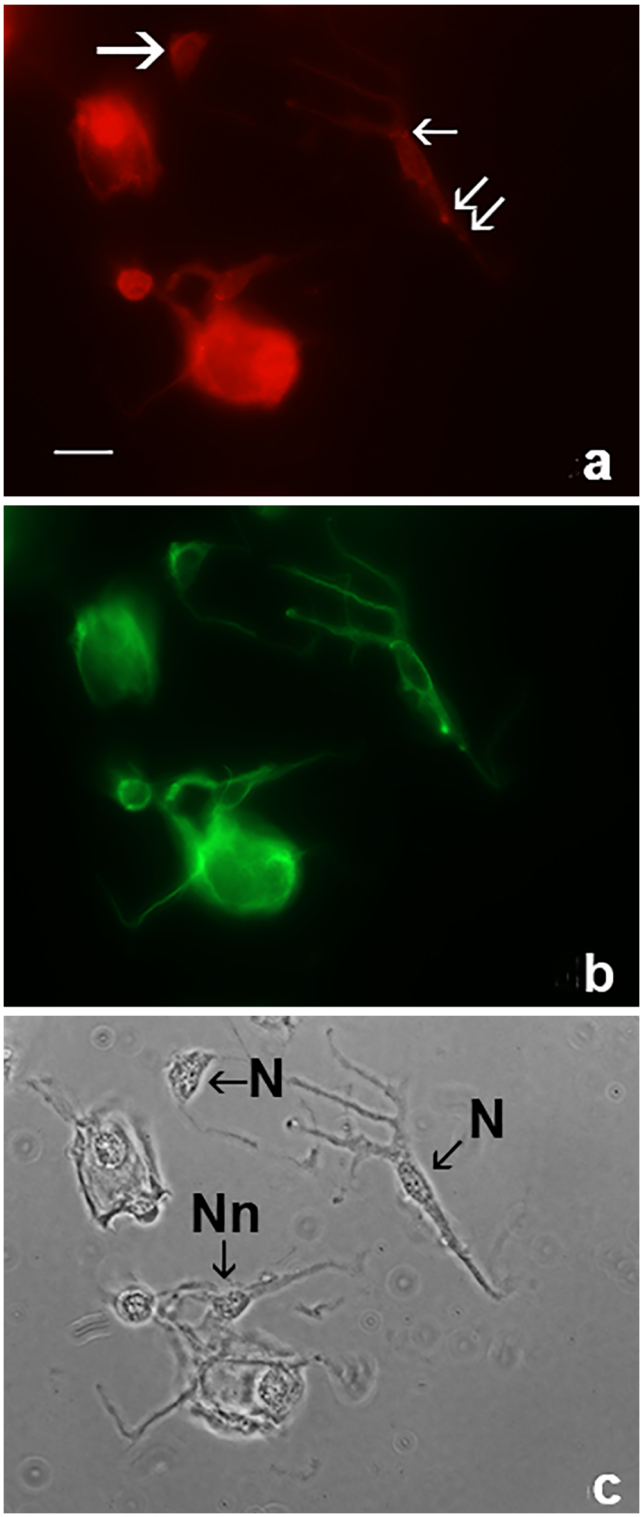

Fig. 8 Two isolated nerve cells (N) and a cluster of nerve cells within a portion of the nerve net (Nn) from the lower body region. Microtubules, labeled with anti-tubulin antibody, help to define the nerve cell bodies and their processes (b). Note disseminated punctate anti-glyR labelling of the neuronal cell bodies and bright patches along a major neurite of the cell on the right as well as at the junction of the cell body and the trifurcating neurites (a, small arrows). There is a moderately bright perinuclear region on the cell in the upper left (a, large arrow). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

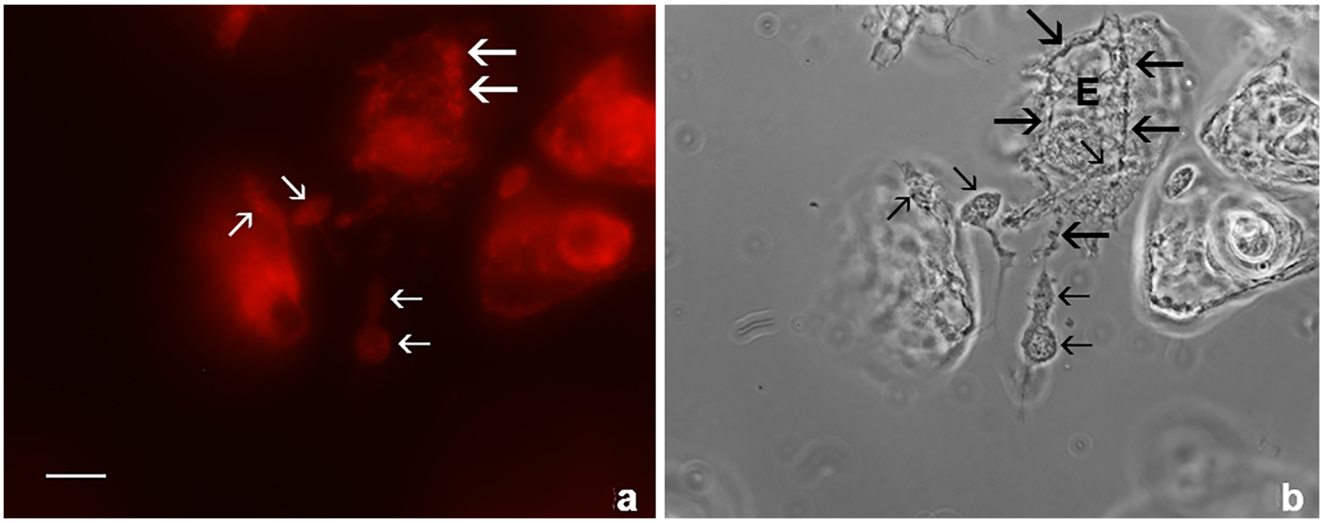

Fig. 9 A portion of the ectodermal nerve net from the upper region of the body associated with EMCs. Note the cell bodies of several morphologically different types of neurons (b, small arrows), of which three are clearly labeled with anti-glyR antibody (a, small arrows). Diffuse and patchy labeling is evident on the nerve cell bodies. A neurite emanating from the lower cell on the right forms an extensive loop (b, large arrows) associated with an EMC (E) at the top of the image. Dense varicosities along the neurite (b) are associated with patches of label along the surface of the EMC (a, large arrows). **a** Texas Red, **b** phase contrast. Bar = 10 μm

numbers from 1 to 26. The first six proteins, two of which we now identify as putative glycine receptor proteins, were identified in the published database as GABA receptor proteins. Proteins #7 (XP_012561154.1) and #8 (XP_012563985.1) were identified in the published database as GlyRα2-like proteins. Among proteins #9 through #26, 11 were previously identified as GABA receptor subunits, including #9 (XP_012556838.1) and #18 (XP_012555765.1), which we further analyzed with respect to their GlyRα1 homology.

We blasted the predicted hydra protein database using the human GlyRα1 strychnine-binding region (AA187–231). Only five proteins showed significant homology. Among these were proteins #1 (XP_012560050.1) and #2 (XP_012560049.1); we designated the other three proteins A, B and C. There is evidence that the human strychnine-binding region is characterized by a functionally necessary GY motif (Gly160, Tyr161; see Fig. 1) (Vandenberg et al. 1992; Lynch 2004). Since neither A, B, nor C had this motif nor did they appear in our original blast search using the entire human GlyRα1 protein, we discounted them in our analysis. Despite the presence of a GY motif in proteins #9 and #18, these were not identified as potential strychnine-binding proteins through our blast with the human strychnine-binding region. Proteins #7 and #8 (identified in the database as GlyRα2-like proteins) were also not recognized.

We blasted the immunogen sequence (AA 178-199) against all predicted hydra proteins. Only four hydra proteins with regions having significant alignment to the immunogen sequence were identified: #1, #2, #9, #18. Based on e-values, the order of decreasing homology was #9, #18, #1 and #2. However, within the regions of homology, the order of decreasing identity was #1, #2, #9 and #18. Individual alignments of the immunogen sequence with hydra proteins #1, #9 and #18 were also prepared (not shown). There were 15 identical amino acids for protein #1, 11 for protein #9 and 10 for protein #18. There were 28 similar amino acids for proteins #1 and #9 and 29 for #18.

Upon BLAST alignment of the human GlyRα1 protein with proteins #1, #2, #9 and #18, e-values of $6e^{-69}$ and $8e^{-69}$ were found for proteins #1 and #2, respectively; e-values of $3e^{-53}$ and $7e^{-30}$ were found for proteins #9 and #18, respectively. Within the regions of strongest homology of the human GlyRα1 protein with proteins #1 and #2, identities of 31% and similarities of 52% were obtained in both cases. When we aligned the human GlyRα1 protein sequence with hydra proteins #1 and #2, using EMBOSS Matcher, a 285 amino acid long region of strong homology was found in both cases and in both cases, there was 35.8% identity and 59.3% similarity within this region (alignments not shown). Proteins #1 and #2 are similar in length (474 and 479 AA); EMBOSS Needle analysis revealed that they are identical except for very short N-terminal sequences (Fig. S1). We also prepared an additional alignment of the human protein with hydra protein #1 using EMBOSS Needle, which revealed regions of strong homology throughout the lengths of these proteins (Fig. S2). The detailed relationships among all five proteins are shown in a complete Clustal Omega alignment of

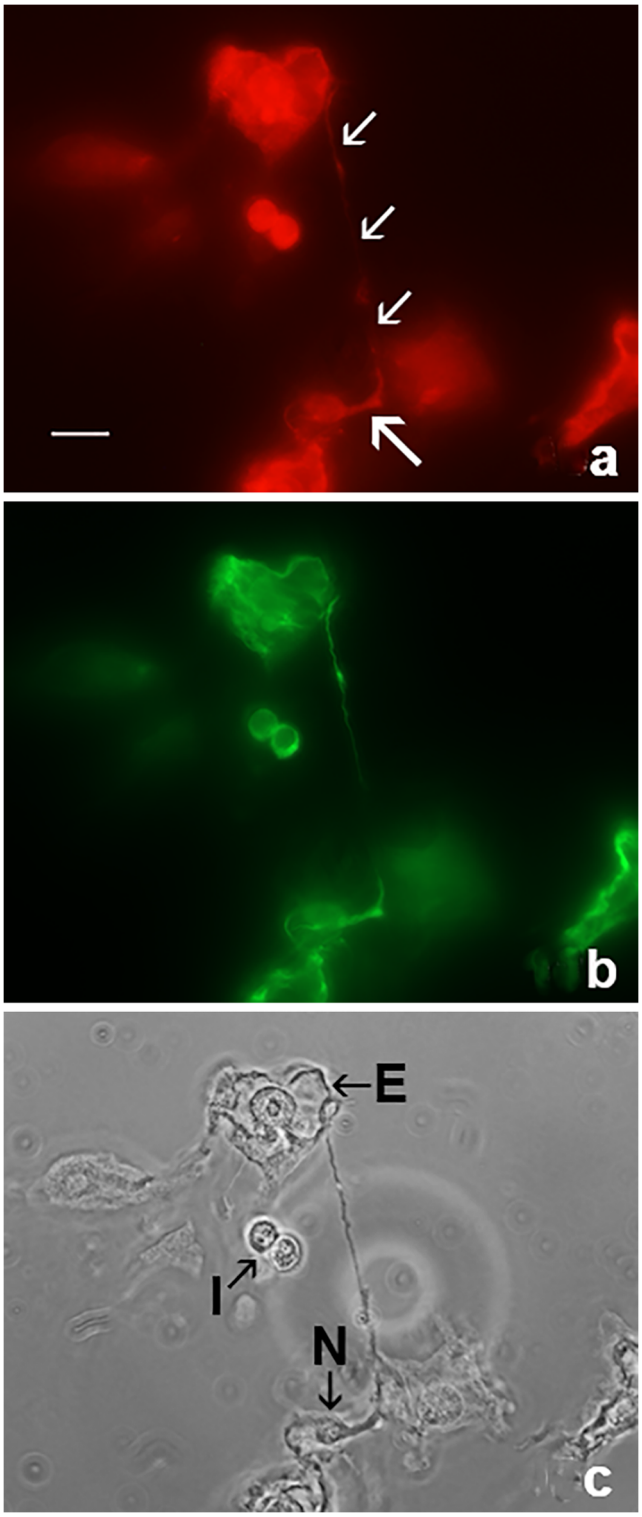

Fig. 10 A portion of the nerve net from the lower third of the body, in which a long neurite extends upward onto a heart-shaped EMC (E) from a nerve cell body (N). Note the moderately diffuse anti-glyR labeling on the nerve cell body and its neurite (a, small arrows); labeling on the neurite is more conspicuous on its thicker, basal portion. (a, large arrow). Also note intense anti-glyR labeling of the cytoplasm of I-cells (I). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

the human GlyRα1 protein sequence and hydra proteins #1, #2, #9 and #18 (Fig. S3).

In order to justify the use of our antibody to identify the glycine receptor in hydra, we prepared a multiple sequence alignment of hydra protein #1 and the strychnine-binding region and immunogen sequence of the human GlyRα1 protein (Fig. 1). This demonstrated the extensive region of homology shared by all three sequences. More importantly, the immunogen and the strychnine-binding region target extensively overlapping portions of the hydra protein. Only four amino acids (KHYN) at the C-terminal end of the human strychnine-binding region are not represented by the immunogen sequence (Fig. 1).

Immunocytochemistry

We observed several different types of anti-glycine receptor antibody labeling on various cells of both the ectoderm and endoderm. The labeling included small and large patches of fluorescence along cell membranes, neurites and myonemes and diffuse labeling in cytoplasm and nucleoli. In double-labeled preparations, anti-α-tubulin labeling was observed in nerve cell bodies and neurites, as well as in the microtubular scaffolds surrounding the cnidocytes and within epitheliomuscular cells.

Epitheliomuscular cells Small and large patches of labeling were seen on the cell surface and myonemes of both endodermal and ectodermal cells in the peduncle and head region. Bright diffuse labeling of nucleoli was evident on many of the cells and disseminated punctate labeling occurred in the cytoplasm, often in the vicinity of nuclei (Figs. 2 and 3). The patches of labeling on the membranes of ectodermal epitheliomuscular cells (EMCs) were frequently in rows, often aligned with the neurites of the nerve net (Fig. 4). Labeling of ectodermal and endodermal cells was especially clear in the peduncle (Figs. 5 and 6). Ectodermal battery cells of the tentacles displayed comparable labeling. Numerous patches of labeling were observed along the battery cell myonemes, particularly near their junctures with the cell body (Fig. 7).

Nerve cells Fluorescent labeling was observed in cell bodies, neurites and nuclei of both ectodermal and endodermal neurons in all regions examined. Labeling was frequently diffuse, although occasional patches on cell bodies and neurites were evident. Labeling was most apparent in or on the surface of nerve cell bodies (Figs. 8 and 9), whereas labeling of neurites could most easily be detected on thicker fibers (Fig. 10).

Differentiating and early developing interstitial cells Various stages of developing interstitial cells (I-cells), including neuroblasts and cnidoblasts, displayed labeling. In early

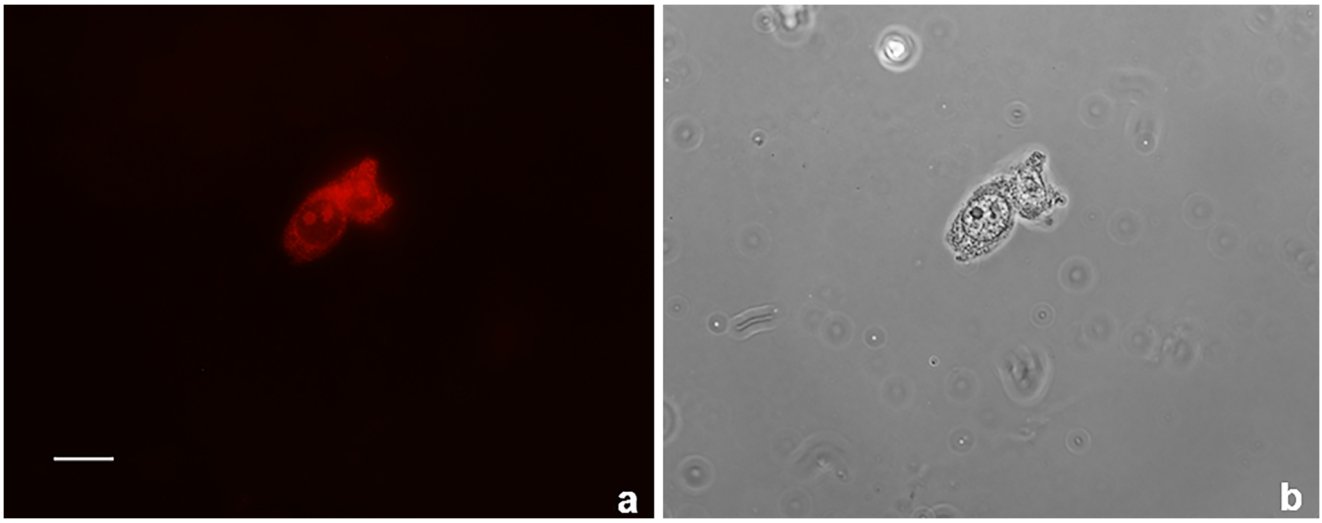

Fig. 11 Undifferentiated I-cells from the "head" region. Note intense small disseminated punctate labeling of the cytoplasm and nucleoli. **a** Texas Red, **b** phase contrast. Bar = 10 μm

undifferentiated I-cells, diffuse, intense labeling was evident in the cytoplasm and nucleoli (Figs. 10 and 11). In differentiated neuroblasts, labeling of nuclei and cytoplasm was evident (Fig. 12). In developing cnidoblasts, depending upon the stage of development, strong labeling was evident in the cytoplasm, nucleoli and nucleoplasm (Figs. 13, 14, and 15).

Cnidocytes

In mature cnidocytes, intense labeling was observed in the region surrounding the cnidocyst capsule in stenoteles, desmonemes and isorhizas (Figs. 16 and 17). Diffuse labeling of nuclei was also apparent.

Battery cell complexes of the tentacle Battery cell complexes consist of the battery cell, encasing the three types of cnidocytes, one or two sensory cells and a ganglion cell, whose neurites extend beyond the battery cell, along the length of the tentacle (Hufnagel et al. 1985). Intense labeling of the interior of the complex was observed (Fig. 16). In addition to labeling of the battery cell cytoplasm, nucleus and myonemes (see above, Fig. 7), intense labeling was apparent in the pericapsular regions of the cnidocytes and in the cell bodies and neurites of the ganglion cells (Fig. 16).

Discussion

In this study, we presented immunocytochemical evidence for a strychnine-sensitive glycine receptor in hydra. We found labeling on the following: cell bodies and neurites of nerve cells, cnidocytes and epithelial cells of both the ectoderm and endoderm of the tentacles and upper and lower thirds of the body column. Differentiating interstitial cells (neuroblasts and cnidoblasts) were also labeled. Labeling of ectodermal and endodermal epithelial cells was pronounced in the cytoplasm, on the myonemes and in some of the nucleoli. Labeling appeared to be patchy (cell membranes, myonemes and neurites), punctate (cytoplasm of endodermal epithelial cells), or uniformly distributed (cytoplasm, nucleoli and cnidocytes). Although all cell types were labeled in our study, not all cells of each type were labeled. In the present paper, we only show images of the cells that were strongly labeled. Our images reveal that the labeling of specific structures within a cell type was consistent and that labeling within each cell was not uniform.

Because we were unable to obtain a specific blocking peptide, we relied on genomic analysis to identify a suitable antibody for hydra's GlyRα1 receptor. The antibody we used was chosen because it was raised against residues 178–227 of the human α1 subunit of the glycine receptor. This region overlaps the major portion of the specific strychnine-binding region in the human GlyRα1 subunit (Vandenberg et al. 1992). In our genomic analysis, we found that the immunogen against which our chosen antibody was made recognized only four proteins in a blast of the *H. vulgaris* protein database. These proteins (#1, #2, #9 and #18) were originally identified as β2-like $GABA_A$ receptor subunits in the NCBI protein database. Although these proteins were identified as $GABA_A$-receptor proteins, our analysis indicated that at least two of them are glycine-receptor proteins.

Our blast using the entire human GlyRα1 protein sequence identified a large number of proteins with homologous sequences, of which proteins #1 and #2 had the strongest

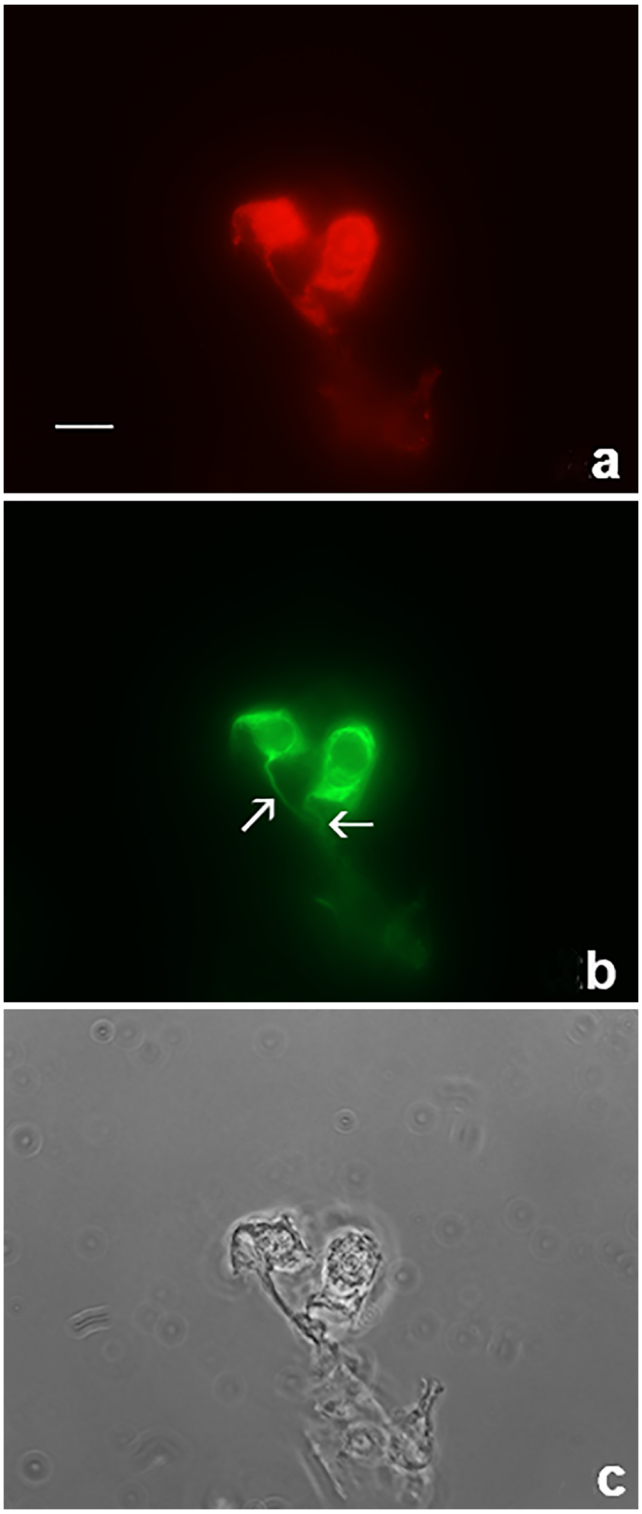

Fig. 12 Early differentiating cells from the lower body region, exhibiting abundant anti-tubulin labeling in their perinuclear cytoplasm and in associated neurites (b arrow). Anti-glyR labeling is evident in the cytoplasm of both cells and the nucleolus of the cell on the right (a). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

homology to the human glyRα1 subunit. A protein blast using the human immunogen sequence identified four proteins (#1, #2, #9 and #18) as possible targets for the antibody we used. Our blast using the human GlyRα1 strychnine-binding region identified proteins #1 and #2 but not #9 and #18, as having a strong likelihood of binding strychnine (it should be noted that proteins #1 and #2 are essentially identical except for small end-terminal regions). Therefore, we conclude that proteins #1 and #2 are likely to be glycine receptor subunits, since the binding of strychnine to the receptor is considered to be a defining characteristic of the glycine receptor studied in other organisms (Vandenberg et al. 1992). In concert with this analysis are the findings of Anctil (2009), in which a single putative but distinct, glycine receptor sequence was reported for the sea anemone, *Nematostella vectensis*.

While our blasts with the entire human glyRα1 and the immunogen also recognized proteins #9 and #18 (originally designated as β2-like $GABA_A$ receptor subunits) because our blast with the strychnine-binding region failed to recognize these proteins, we consider them unlikely to be glycine receptor proteins, despite the fact that proteins #9 and #18 have a greater probability of binding our antibody than proteins #1 and #2 and despite the presence of the GY motif in #9 and #18, which is necessary but not sufficient for strychnine binding (Vandenberg et al. 1992).

Thus, given our genomic analysis and the fact that in the NCBI database #9 and #18 are identified as $GABA_A$ receptor subunits, we cannot discount the possibility that our antibody labeled both glycine and $GABA_A$ receptor subunits in our immunocytochemistry experiments. This is especially possible since we were unable to obtain a blocking peptide for our antibody and since GABA and glycine receptors belong to the Cys-loop LGIC superfamily, where a high degree of overlapping sequences should be expected. Further, pharmacological, physiological and behavioral experiments revealed the inhibitory effects of GABA reception in hydra and $GABA_A$R subunits have been immunochemically identified in hydra (Pierobon et al. 1995; Kass-Simon et al. 2003; Concas et al. 2016; Lauro and Kass-Simon 2018). The recent study by Concas et al. (2016) provided immunohistochemical evidence that several $GABA_A$ receptor subunit isoforms, namely α3, β1, γ3 and δ, are differentially distributed and differently co-localized in hydra's body regions. The authors suggested that in "hydra, GABA receptors are heterologous multimers, possibly subserving different physiological activities."

Nonetheless, for reasons given above, our immunohistochemical observations appear to comport with earlier physiological and biochemical findings indicating inhibitory glycine reception in hydra. Unfortunately, we are unable to directly compare our immunohistochemical findings with

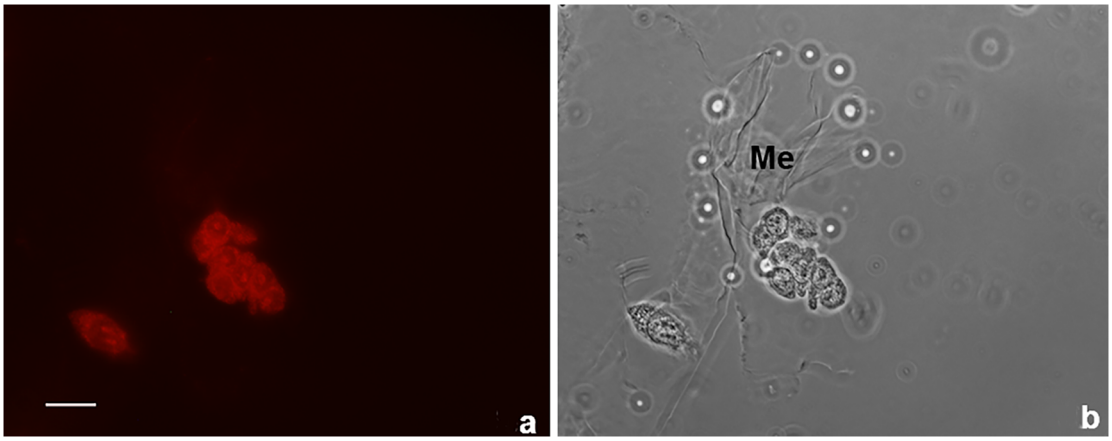

Fig. 13 Cluster of developing I-cells, associated with a piece of cellophane-like mesoglea (Me), from the lower body column. There is distinct anti-glyR labeling of the cytoplasm and nucleoli in all cells. **a** Texas Red, **b** phase contrast. Bar = 10 μm

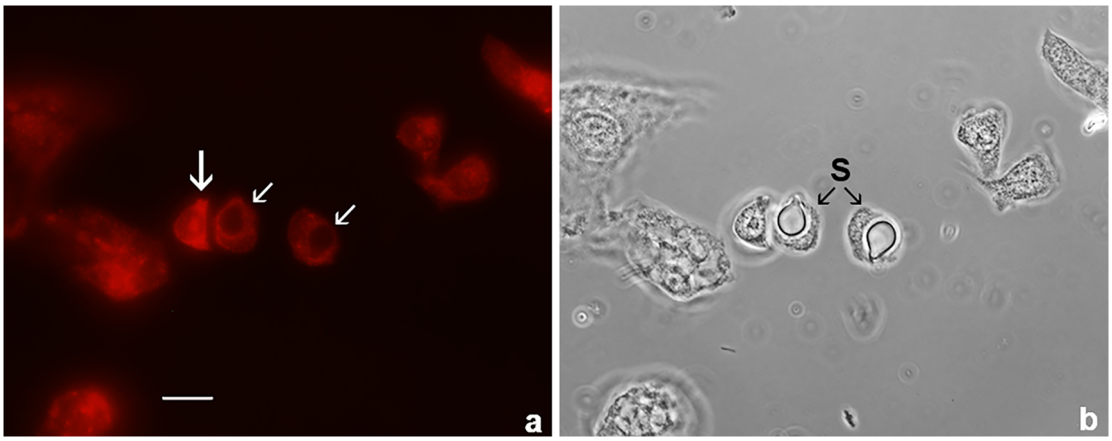

Fig. 14 Developing late-stage stenotele cnidoblasts (S) from the hypostomal region. Note the prominent anti-GlyR label in the nucleolus and cytoplasm in the cell adjacent to one of the two cnidoblasts (a, large arrow); in the cnidoblasts, labeled nucleoli are not apparent and cyctoplasmic labeling is disseminated and punctate (a, small arrows). **a** Texas Red, **b** phase contrast. Bar = 10 μm

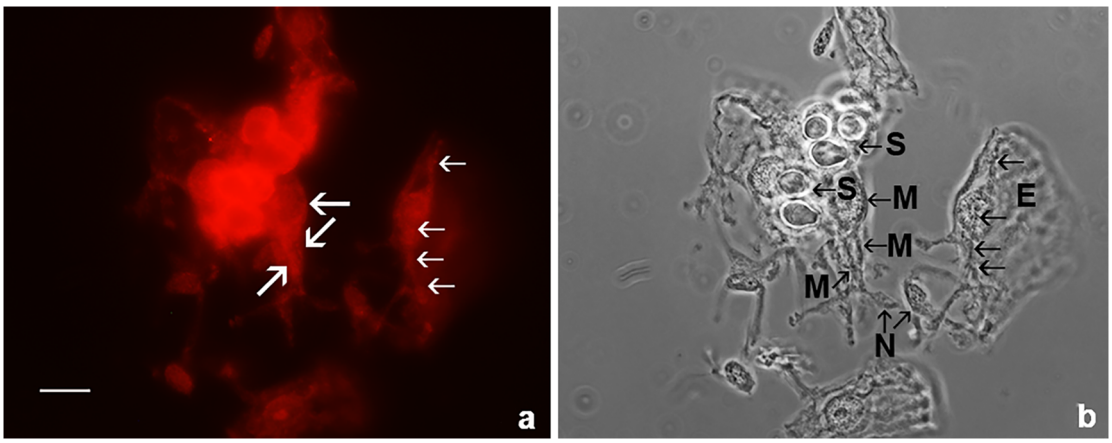

Fig. 15 A nest of late-stage stenotele nematoblasts (S) from the lower body region. Intense pericapsular anti-glyR labeling is apparent. Note patchy labeling associated with portions of the nerve net (a, b, small arrows), attached to an ectodermal EMC (E). Other neurites appear attached to labeled myonemes (M) of the EMCs (a, large arrows). Note that two nerve cell bodies (a, b, N) are also labeled. **a** Texas Red, **b** phase contrast. Bar = 10 μm

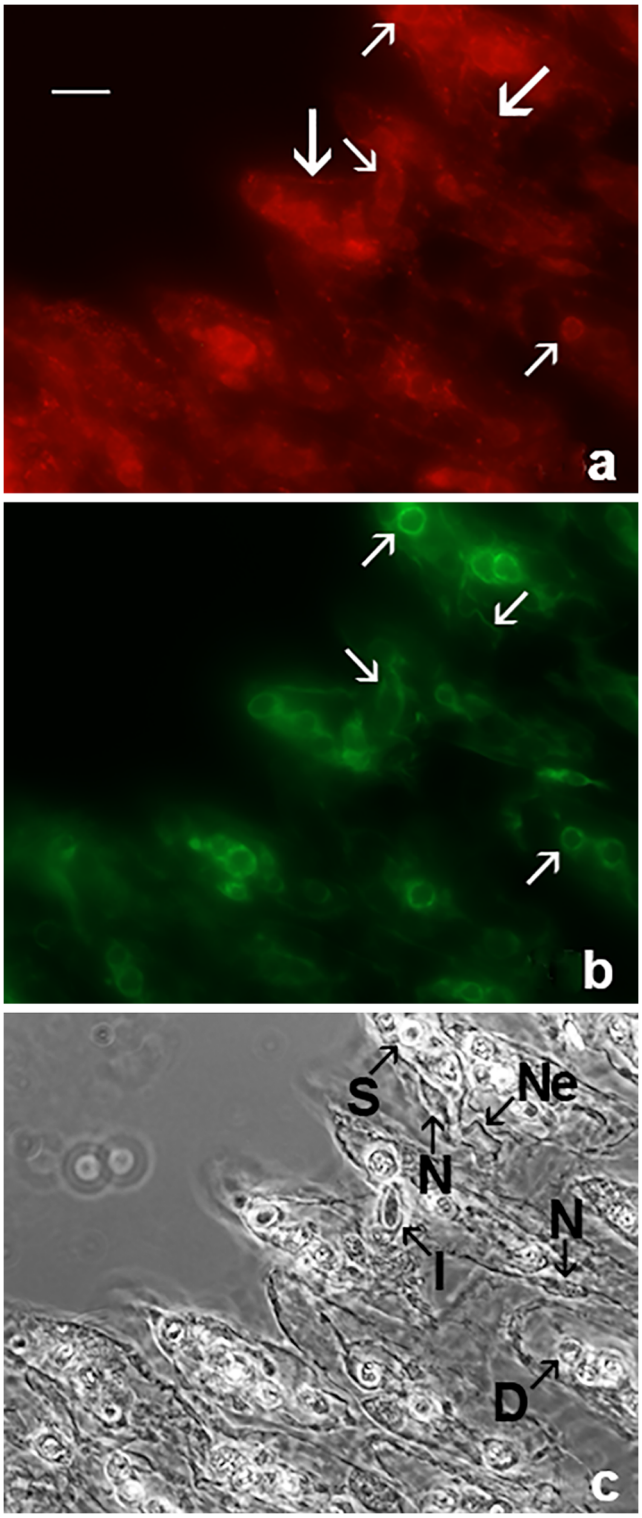

Fig. 16 Battery cell complexes in a portion of a tentacle. Note intense, continuous anti-alpha-tubulin labeling around the capsules of stenotele cnidocytes (S) and along neurites (Ne) and the moderate labeling around the capsules of isorhizas (I) and desmonemes (D) (b, arrows). A corresponding pattern of patchy anti-glyR labeling is evident on the cnidocytes (a, small arrows). Note the continuous and patchy anti-glyR labeling of neurites extending across battery cells (a, large arrows) and the diffuse labeling of two nerve cell bodies (N). **a** Texas Red, **b** Alexa 488, **c** phase contrast. Bar = 10 μm

those of Concas et al. (2016) because our experiments were performed on dissociated cells and not on intact animals. During hydra's feeding response, glycine increases the duration of mouth opening that is counteracted by the application of strychnine, which suggests an inhibition of the contractile elements of hypostomal epithelial cells by glycine (Pierobon et al. 2001; Pierobon 2015). This view corresponds to our present observation that the myonemes of both ectodermal and endodermal cells of the head region have extensive patches of GlyR labeling.

Our electrophysiological experiments (Ruggieri et al. 2004) indicated that a strychnine-sensitive glycine receptor is involved in the inhibition of rhythmic potential impulses that are generated by pacemakers controlling the elongation of the body column (Passano and McCullough 1965; Kass-Simon and Passano 1978). Our present study shows that the myonemes of endodermal epithelial cells exhibit positive immunoreactivity, consistent with an expected inhibition by glycine of the impulses associated with body elongation and its antagonism by strychnine. We also found that the output of the ectodermal body contraction pacemaker system is reduced by both glycine and strychnine (Ruggieri et al. 2004). These findings correlate with our present observation that the myonemes of ectodermal epithelial cells and the ectodermal nerve net label positively with our antibody.

In the nervous system, in addition to nuclear and cell body labeling, strongly labeled patches corresponding to numerous conspicuous varicosities on the neurites were evident on coincident areas of EMC membranes. The varicosities presumably correspond to presynaptic sites of transmitter release (Nishida et al. 2017). The close association of nerve and effector membrane labeling suggests that these patches represent areas of functional interaction.

Bright labeling was found in the cytoplasm surrounding the cysts of all cnidocytes but was most pronounced in stenotele cnidocytes. Frequently, labeling was distinctly pericapsular; in some instances, it appeared as asymmetric patches on either side of the capsules. Similar patterns of labeling were observed in previous studies using anti-NMDAR antibody (Scappaticci et al. 2004). This localization was considered to coincide with the behavioral/physiological finding that NMDA receptors were involved in the control of stenotele discharge.

Since proteins #9 and #18 have been imputed to be $GABA_A$ β2-like receptors and since Concas et al. (2016) identified anti-$GABA_A$-β1-like receptor labeling in the tips of the tentacles, it is possible that our antibody identified a $GABA_A$ receptor in the tentacles. To date, no behavioral/physiological correlation with either the $GABA_A$ receptor or the strychnine-sensitive glycine receptor has been identified in the tentacles. However, based on our present immunohistochemical and genomic analysis, the presence of glycine receptors in the tentacles should remain a possibility.

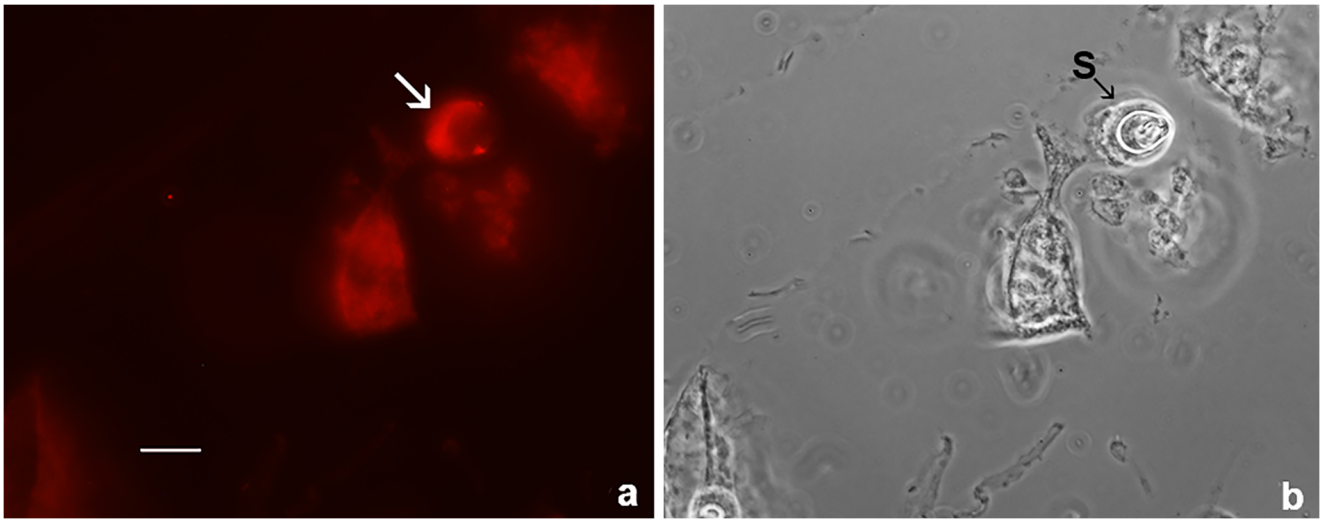

Fig. 17 Intense pericapsular labeling (a, arrow) of a stenotele cnidocyte (S) associated with ectodermal cells of the head region. **a** Texas Red, **b** phase contrast. Bar = 10 μm

In our present study, labeling was intense in the nucleoli of some I-cells and endodermal and ectodermal EMCs. In an earlier publication (Kass-Simon et al. 2009), we described nucleolar labeling with an anti-NMDA receptor antibody in developing I-cells and endodermal EMCs, which we interpreted as evidence for a possible shuttle mechanism between nucleus and cell membrane or the deployment of alternative splice variants to different cellular locations. However, because nucleolar labeling in the present case was found among several types of cells and, unlike in the NMDA experiment, the corresponding peptide was not available, we cannot specify the nature of the nucleolar labeling.

Among differentiated and undifferentiated I-cells, labeling was seen in many stages of development, including in clusters of early undifferentiated cells and late immature stenoteles and neuroblasts. However, the occurrence of extensive labeling in progenitor cells should not be considered contradictory to the more restrictive labeling found in mature cells, since during development, gene expression becomes compartmentalized (cf. Salvador-Martinez and Salazar-Ciudad 2015).

Because glycine receptors have been studied primarily in mammalian nervous systems, little information about their occurrence and function in the invertebrates has been available (cf. Chapman et al. 2010; Putnam et al. 2007). In our study, the conspicuous presence of immuno-positive labeling throughout hydra's ectodermal and endodermal neuro-effector systems, together with genomic data, give further evidence that glycine inhibition is a component of hydra's behavioral coordinating systems. Thus, the finding that putative GlyR proteins exist in hydra, an organism positioned near the base of the metazoan evolutionary tree, suggests that glycine receptors may be as widely present in the invertebrates as in the vertebrates.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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