

The two nerve rings of the hypostomal nervous system of *Hydra vulgaris*—an immunohistochemical analysis

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Abstract In *Hydra vulgaris*, physiological and pharmacological evidence exists for a hypostomal circumferential neuro-effector pathway that initiates ectodermal pacemaker activity at tentacular-hypostomal loci coordinating body and tentacle contractions. Here, we describe an ectodermal nerve ring that runs below and between the tentacles, and an anti-GABA_B receptor antibody-labeled ring coincident with it. The location of this ring is consistent with the physiology of the hypostomal pacemaker systems of hydra. We also describe a distally located, ectodermal ring of nerve fibers that is not associated with anti-GABA_B receptor antibody labeling. The neurites and cell bodies of sensory cells contribute to both rings. The location of the distal ring and its sensory cell neurites suggests an involvement in the behavior of the mouth.

Between the two rings is a network of anastomosing sensory and ganglion cell bodies and their neurites. Phase contrast, darkfield, and antibody-labeled images reveal that the mouth of hydra comprises five or six epithelial folds whose endoderm extensively labels with anti-GABA_B receptor antibody, suggesting that endodermal metabotropic GABA receptors are also involved in regulating mouth behavior.

Keywords Sensory cells · Feeding behavior · Pacemakers · Neuroreceptors · GABA_B

Introduction

Circumferential nerve rings that conduct impulses from body and tentacle pacemaker systems exist in all cnidarian classes (Hertwig and Hertwig 1879; Bullock and Horridge 1965; Garm et al. 2006, 2007; Kass-Simon and Hufnagel 2015). In hydrozoans, the nerves of the ring are generally found encircling the hypostome in polyps or the edge of the bell in medusae (Singla 1978a, 1978b). Circumhypostomal nerve rings have been found in several species of the genus *Hydra*. In histological sections of *H. pirardi* and *H. pseudo-oligactis*, Davis et al. (1968) reported the presence of a neural ring at the base of the hypostome and tentacles. In *Pelmatohydra robusta*, an electron microscopic analysis of ultrathin sections revealed that bundles of neurites, emanating from clusters of ganglion cells, form a ring near the periphery of the hypostome, just above the tentacles (Matsuno and Kageyama 1984). Immunohistochemical studies of *H. oligactis* and *H. littoralis* labeled with JD1 (a monoclonal antibody specific for hypostomal sensory cells) have revealed a thin continuous supra-tentacular circumhypostomal nerve ring (Dunne et al. 1985; Yu et al. 1985). A similar nerve ring was also observed in *H. oligactis*, *H. robusta*, and *H. circumcincta* labeled with

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anti-RFamide and other anti-peptide antisera but was not seen in similarly labeled *H. vulgaris* (formerly *H. attenuata*) or in *H. magnipapillata* (Grimmelikhuijzen 1985; Grimmelikhuijzen et al. 1989; Takahashi et al. 2003; Koizumi et al. 1992, 2004; Koizumi 2007).

The typical behavior of *H. vulgaris* is controlled by four pacemaker systems: (1) the ectodermal contraction burst (CB) system, whose activity is responsible for the longitudinal contraction of the body column (Passano and McCullough 1964), and whose impulses generally arise from pacemakers that were electro-physiologically found to be at or near the insertion of the tentacles (Kass-Simon 1972, 1973), (2) the tentacle pulse (TP) system, responsible for tentacle contraction, whose pacemakers are located in individual tentacles (Rushforth and Burke 1971), (3) the endodermal rhythmic potential (RP) system, whose impulses control the extension of the body column (Passano and McCullough 1964, 1965; Kass-Simon and Passano 1978), and (4) the neck pulse (NP) system, localized to the distal body column. The TP system is considered to induce body contractions by excitation of the contraction burst system (Rushforth and Burke 1971; Kass-Simon 1972, 1973). The impulses of the CB system, which arise near the insertion of the tentacles, are transmitted around the hypostome, induce tentacle contractions, and coordinate the contraction of the body column (Kass-Simon 1972, 1973).

Although circumhypostomal nerve rings have been described in several species of hydra, and although the physiological evidence indicates that a similar ring should exist in *H. vulgaris*, previous morphological studies have failed to identify such a ring in this species. Here, we present immunohistochemical evidence for the existence of two circumhypostomal nerve rings in the ectoderm of *H. vulgaris*: a proximal nerve ring that interconnects with tentacular nerves at loci situated at the bases of the tentacles, and a distal nerve ring located in the dome of the hypostome, above the tentacle ring. In concordance with previous studies (Kinnamon and Westfall 1981a, 1981b, 1984; Grimmelikhuijzen 1985; Koizumi et al. 1992), we observed numerous sensory cells surrounding the area in which a mouth opening forms and contributing their neurites to the two rings.

Although many studies have reported the existence of neuropeptide-containing neurons in the hypostome (e.g., Grimmelikhuijzen 1985; Koizumi et al. 1992), no studies have been published describing classic neurotransmitters or their receptors in this region. Here, we describe the distribution of gamma-aminobutyric acid B (GABA_B) receptors in relation to the ectodermal nerve net and also provide evidence that the hydra's oral cavity is surrounded by five or six tissue folds within which is a GABA_B-receptor-rich endoderm.

This work was initiated as part of a collaboration between the laboratories of A. Concas, L. A. Hufnagel, G. Kass-Simon, and P. Pierobon. Preliminary reports of these findings have been presented at International Workshops on Hydra

Development and Multicellular Evolution, in Tutzing, Germany (2009, 2011, 2013) and at the Society for Neurosciences Annual Meeting (2010).

Materials and methods

Culturing methods All experiments were carried out on *Hydra vulgaris*, maintained in glass culture dishes in modified M solution (Muscatine and Lenhoff 1965), containing NaHCO₃ (1×10^{-3} M), CaCl₂ (2.5×10^{-4} M), MgCl₂ (5×10^{-4} M), and EDTA (1×10^{-5} M) in distilled water (pH 7.2 ± 0.1) at 19 °C. Animals were fed with *Artemia salina* nauplii on alternate days. The culture solution was changed 1 h after feeding.

Immunohistochemical methods For immunohistochemistry, 48 h starved non-budding hydra were relaxed on agar-coated slides by using dilute (1/1000) menthol in BVC solution and transected directly beneath the tentacles; the tentacle/hypostome portions were retained. All subsequent steps were conducted on whole mounts on slides. To promote later antibody penetration, some tentacle/hypostome portions (heads) were treated for 1–3 min with dissociation medium (glycerol: acetic acid: deionized water 1:1:7), modified from David (1973) in order to partially dissociate the cells. Treated heads were immediately fixed with Lavdowsky's fixative (Dunne et al. 1985) for 1 h. After washes with 10 mM phosphate-buffered saline (PBS) and 10 mM modified PBS (MPBS) containing 1 % bovine serum albumin (BSA), 0.2 % Tween 80, 0.05 % NaAzide, 0.1 % polyethylene glycol 20, and 0.02 % KCl (MPBS/BSA; Erskine 1989), preparations were incubated at room temperature overnight in primary antibody: either mouse anti-sea urchin α -tubulin monoclonal antibody (mAb, Sigma Clone B512) at 1/1000 in MPBS/BSA or a combination of anti- α -tubulin (1/1000) and rabbit anti-rat GABA_B R1 polyclonal antibody (pAb, Chemicon AB5850; immunogen: N-terminus synthetic peptide) at 1/100 in MPBS/BSA. The GABA_B R1 antibody was selected based on our genomic analysis. Use of Clustal W2 (www.ebi.ac.uk/Tools/msa/clustalw2/) and an alignment comparison of the immunogen with a subunit 1-like protein of *H. vulgaris*, XP_012556198.1, identified regions of strong homology, which had 20 % identity and 70 % similarity (Fig. S1). We also performed a pair-wise alignment of the immunogen with subunit 1 and subunit 2 of the GABA_B R2, XP_012555926 of *H. vulgaris*, using EMBOSS matcher (www.ebi.ac.uk/Tools/psa/emboss_matcher/; Fig. S2). Whole-mounts were washed with MPBS/BSA and treated for 1–24 h with secondary antibodies. For samples labeled only with anti- α -tubulin antibodies, a 1/400 or 1/500 dilution of goat anti-mouse IgG secondary antibody, tagged with Alexa 488 or Texas Red (Molecular Probes/Invitrogen) was used. For samples double-labeled with anti- α -tubulin mAb and anti-GABA_B R1 pAb, we employed a mixture of Alexa-488-tagged goat anti-mouse IgG (1/400) and

Texas Red-tagged goat anti-rabbit IgG (1/400, Molecular Probes/Invitrogen). After antibody treatments, the tissue was washed and covered with Prolong Gold anti-fade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Coverslips were applied and stabilized with nail polish. The slides were stored at -20°C and examined and photographed within 1–2 weeks. Digital photographs of phase contrast and fluorescence images were taken at a resolution of 1300×1030 pixels with a Zeiss AxioPlan 2 imaging system equipped with epifluorescence filters and a Zeiss AxioCam color digital camera. All slides in a given experiment were photographed under the same conditions, with identical exposure times.

For the analysis of the results, brightness and contrast adjustments were applied equally to control and experimental images with Photoshop (Adobe Systems). All images selected for publication show fluorescence labeling above background. Nerve cell bodies and their processes were identified by their strong labeling with anti- α -tubulin antibody. Although, in our whole-mount preparations, we were unable to observe cilia on/in the various cells, we identified putative ganglion and sensory cells by their locations and typical morphologies. Thus, ganglion cells are typically multi-polar and located deep within the ectoderm. In contrast, the bodies of sensory cells project to the surface of the ectoderm and are either monopolar or have a triangular cell body whose distal apex is oriented toward the tissue surface (Westfall 1973). We also relied on the many depictions of nerve cells in hydra by other workers (e.g., Hadzi 1909; Burnett and Diehl 1964; Grimmelikhuijzen 1985; Bode et al. 1988).

Negative control slides, in which primary antibody was omitted, were included in all experiments. Specific binding of primary antibodies was demonstrated, because negative control slides revealed only very low background fluorescence that was not detectable under the imaging conditions used for the experimental slides (images not shown).

Results

We have examined the nerve net in whole mounts of intact and partially dissociated hydra heads labeled with anti- α -tubulin mAbs or double-labeled with anti- α -tubulin mAbs and anti-GABA_B receptor pAbs. The hypostome, with the mouth closed and viewed from above, was circular or hexagonal in appearance, with up to five intact or partial tentacles still attached (Fig. 1a). In some cases, all of the tentacles had become detached, and their insertion sites were sometimes marked by the presence of a few tentacle cells attached at the margin of the hypostome (Fig. 1b) or by oval gaps in the anti- α -tubulin-labeled nerve net (Fig. 1b'). The location of the mouth was indicated in darkfield images by a dark star-shaped pattern, outlined by gray unpigmented, presumably ectodermal, tissue at the center of a region in which abundant

orange-pigmented inclusion bodies designated the presence of endodermal tissue beneath the ectoderm (Fig. 1c). In phase contrast images at the level of the ectodermal surface, the mouth opening was not apparent (Fig. 1b). This agrees with earlier descriptions of an absence of an oral opening at the apex of the hypostome (Campbell 1987; Technau and Holstein 1995; Carter et al. 2016); however, at a slightly more proximal focus, the mouth could usually be discerned by the presence of a thin dark line outlining an in-folding of the ectodermal and endodermal tissue (Fig. 1c'). Thus, the mouth appeared to be formed by five to six folds of tissue, emphasized by the arrangement of the endodermal pigment granules (Fig. 1c). In a side view, the ectoderm at the apex of the hypostome formed a continuous layer over the mouth (Fig. 1d). This observation was supported by *en face* phase contrast images taken at the focal plane of the apical surface (Fig. 1e).

Hypostomes labeled with anti- α -tubulin antibodies In fluorescent images of anti- α -tubulin-labeled hypostomes, the location of the mouth was indicated by a well-defined dark region surrounded by labeled sensory cell bodies and radial neurites (Fig. 1b', e'). Ganglion cells and sensory cells and their processes were distributed throughout the ectodermal nerve net of the hypostome (Fig. 1b', e'). Two separate nerve rings were detected with the anti- α -tubulin antibody: the proximal nerve ring and the distal nerve ring (Fig. 2a, a', b, b').

Proximal hypostomal nerve ring The proximal hypostomal nerve ring was identified as a loosely organized belt of mainly circumferentially oriented nerve fibers and cell bodies, located in the ectodermal layer, at the margin between the hypostome and the body column (Fig. 2a–c). The nerve ring was seen to extend between the tentacles and to dip under the tentacle attachment sites (not shown). The topology of the fibers above the tentacle-hypostome junction was different from that below the junction (Fig. 3a, a'). Beneath the tentacle bases, intertwining neurites imparted a ladder-like appearance to the ring (Fig. 3a). Neurites extended between the tentacle and the ring and between the body column and the ring; the ladder was oriented perpendicularly to the axis of the tentacle (Fig. 3a). Above the tentacle bases, prominent nerve bundles were observed to radiate directly between the hypostomal ectoderm and the tentacle ectoderm (Fig. 3a', b, b'). These were continuous with conspicuous, previously described, longitudinal nerve tracts of the tentacle (Hufnagel and Kass-Simon 1988), composed primarily of neurites and bodies of ganglion cells (Fig. 3c). At the lateral margins of the sites of tentacle attachment, the proximal nerve ring contained clusters of nerve cell bodies and tangled neurites (Figs. 3d, 4a, b). Associated with these were clusters of stenotele cnidocytes (Fig. 4b). The lateral nerve clusters were connected by prominent nerve fibers that belonged to the proximal nerve ring and that extended between the tentacles (Figs. 2b, 4c, c'). Sensory

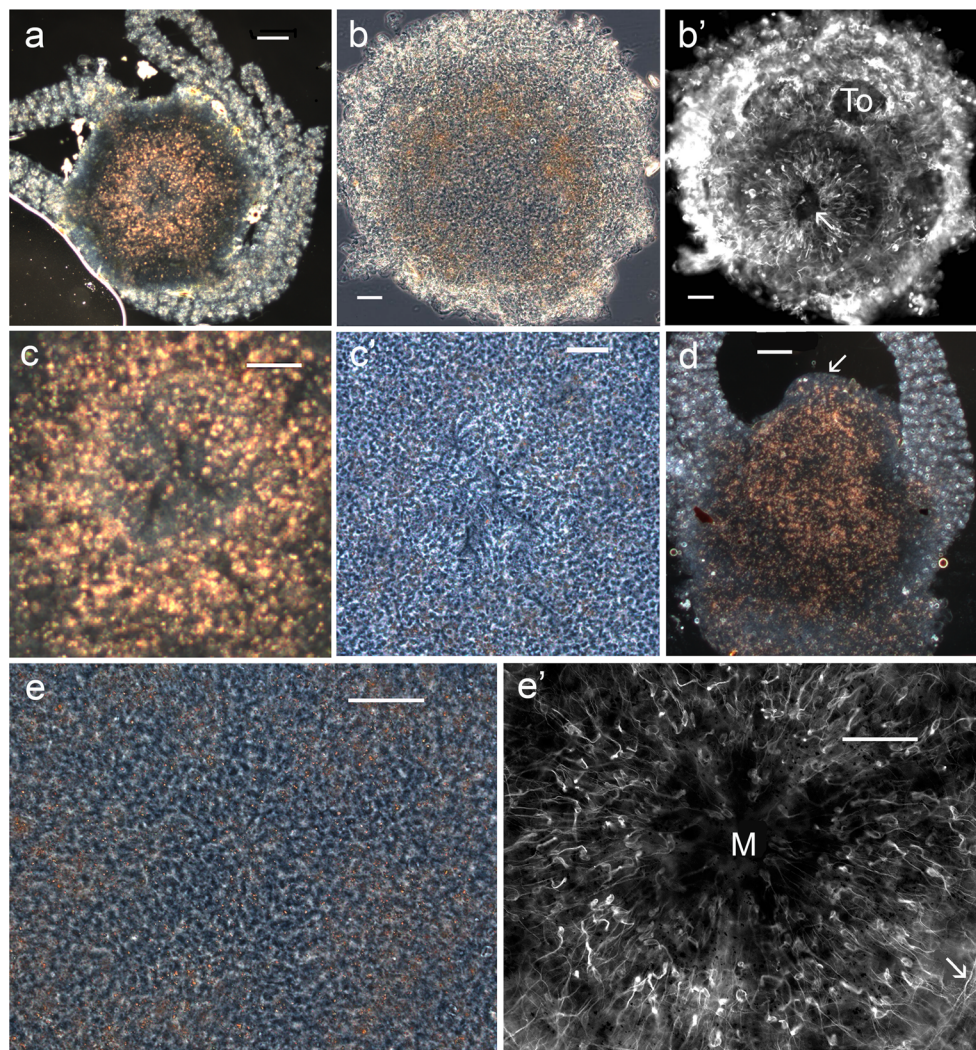


Fig. 1 **a** Isolated hypostome/tentacle preparation of *Hydra vulgaris*, with attached tentacles. Darkfield image. The hypostomal endoderm is marked by the presence of orange inclusion bodies surrounding the mouth. *Bar* 100 μm . **b** Phase contrast image of a hypostome whose tentacles have become detached. *Bar* 40 μm . **b'** Fluorescence image of the same hypostome as in **b** but labeled with anti- α -tubulin antibodies. Tentacle insertion sites are indicated by dark gaps (*To* tentacle opening) arranged circumferentially near the margin of the hypostome. The mouth (*arrow*) is located central to the tentacle insertion sites. *Bar* 40 μm . **c** Portion of the closed-mouth hypostome shown in **a**. Darkfield image of a transverse optical section just beneath the tip of the hypostome. Note, at the center, the star-shaped pattern of possible ectodermal tissue (having few orange inclusion bodies) surrounded by endodermal tissue containing

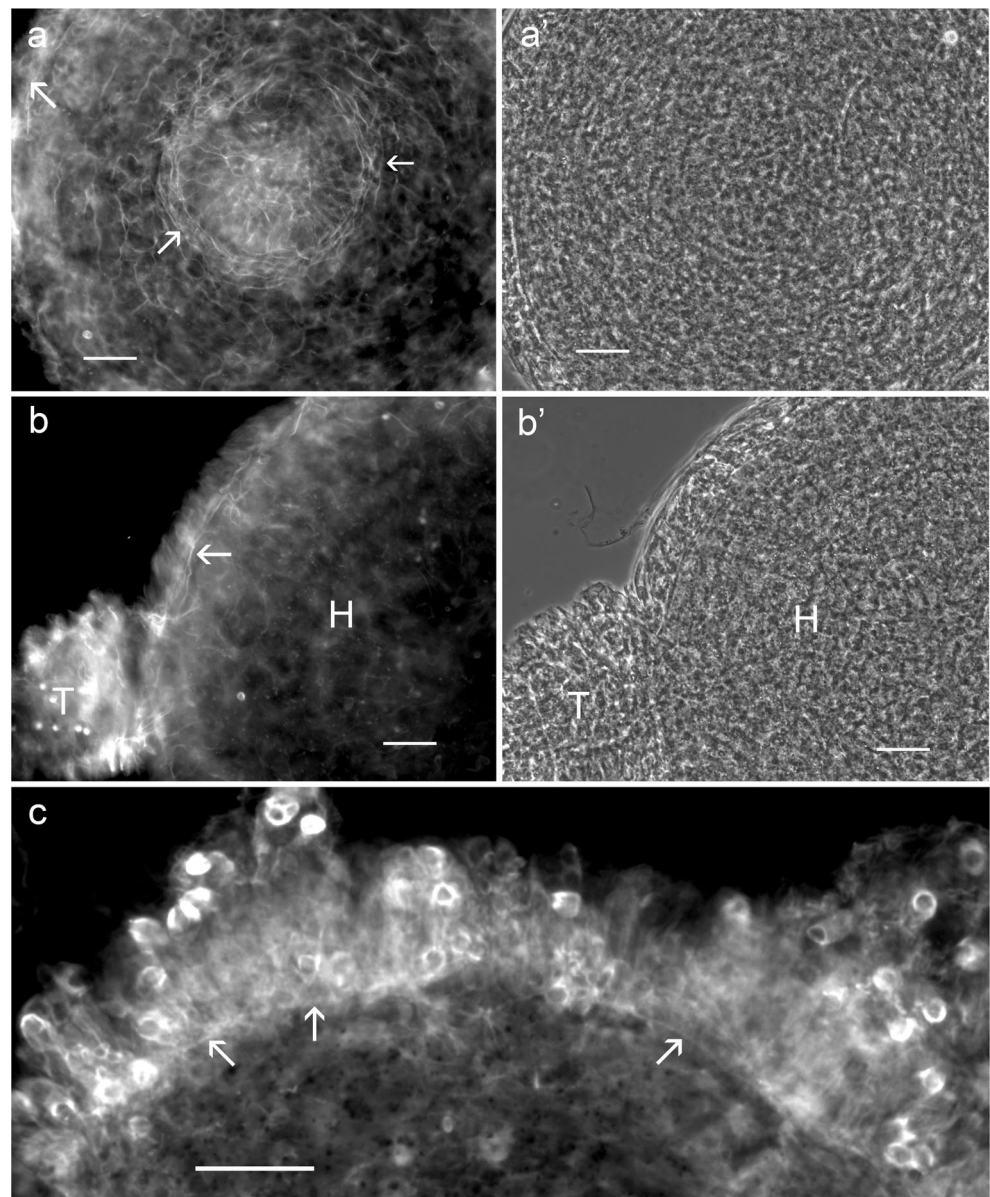
many inclusion bodies. *Bar* 50 μm . **c'** Phase contrast image at the same focal plane as **c**. *Bar* 40 μm . **d** Side view of an ablated hypostome/tentacle preparation focused in a plane that transects the apex of the hypostome. Note the unpigmented ectodermal cells covering the apex of the hypostome (*arrow*). Darkfield image. *Bar* 75 μm . **e** *En face* view of the extreme apex of a hypostome. Phase contrast. Note that the tissue forms an unbroken surface. *Bar* 50 μm . **e'** Fluorescence image of the hypostome shown in **e** but labeled with anti- α -tubulin antibody (taken at the same focal plane as in **e**). Abundant monopolar sensory cells are distributed throughout the apical ectoderm, with their axons oriented away from the mouth (*M*). Note the abundance of sensory cells surrounding the presumptive mouth opening. A portion of the distal nerve ring is seen *lower right* (*arrow*). *Bar* 50 μm

cell bodies located between the tentacles contributed their neurites to the proximal ring (Figs. 4d, e, 5a–a''). The proximal ring, composed of both neurites and cell bodies, consisted of more than one nerve bundle or tract (Fig. 4d, e). As a result of viewing many images, we concluded that each bundle contained the processes of several cells, with neurite interconnections between bundles. Because of the anastomosing and intertwining neurites, the proximal nerve ring of *H. vulgaris*, labeled with anti- α -tubulin antibodies, had the character of a nerve plexus (Figs. 2b, 4d). In summary, the proximal nerve

ring appeared to be coincident with the ring of tentacle attachment sites and directly to connect the nerve and cnidocyte clusters at the bases of the tentacles.

Distal hypostomal nerve ring A second ring was observed midway up the hypostome, above the ring of tentacles. This distal nerve ring was composed of circumferentially oriented nerve fibers and cell bodies (Figs. 1e', 2a, a'). Above the ring were numerous, apically oriented, sensory cells whose processes lay perpendicular to the ring (Figs. 1e', 5b, c, d, d').

Fig. 2 **a** Hypostomal nerve rings of *H. vulgaris*. *En face* view of a single hypostome labeled with anti- α -tubulin antibodies. Fluorescence image. Note the loosely organized fibers of the distal nerve ring (*small arrows*) and portions of the more densely organized proximal nerve ring (*large arrow*). Bar 35 μ m. **a'** Phase contrast image corresponding to **a**. Bar 35 μ m. **b** Fluorescence image of a portion of the proximal nerve ring (*arrow*) near the base of an attached tentacle (*H* hypostome, *T* tentacle). Bar 35 μ m. **b'** Phase contrast image corresponding to **b** (*T* tentacle, *H* hypostome). Bar 35 μ m. Images **a**–**b'** were provided by Crisostomo Gomez. **c** Proximal nerve ring in an anti- α -tubulin-labeled hypostome. Only portions of the ring itself are in focus (*arrows*). Many associated cnidocytes and nerve fibres are also labeled. Bar 126 μ m



The ring comprised mainly of long, circumferentially oriented, cell processes encircling the apex of the hypostome. The distal nerve ring was loosely organized and did not always appear to be continuous (Figs. 2a, 5b, 6a). It lay between the distal region of the ectoderm, an area rich in sensory cell bodies and their processes, and a more proximal region, an area rich in circumferentially oriented nerve fibers (Fig. 2a). Sensory cells of the hypostome contributed to the distal nerve ring (Fig. 5d). Many of their cell bodies were observed in close proximity to the opening of the mouth (Figs. 5b, c, 6b).

Sensory cells of hypostome At least three types of morphologically distinct putative sensory cells were differentially distributed over the hypostome. One type, type A, was characterized by a long thin cell body that was frequently difficult to distinguish from its primary

neurite (Figs. 5b, c, d, d', 6a–c, e, e'). Type A was found only near the mouth opening. The single neurite of the cell appeared to extend deeply into the ectodermal layer (Figs. 5d, 6a–c). Some of the neurites of the type A cells appeared to make contact with the distal nerve ring or to extend past it toward the periphery of the hypostome (Fig. 6a).

The second type of putative sensory cell, type B, had a club-shaped cell body from which a single neurite emanated (Figs. 5a'', 6b, d). Type B cells were distributed over the entire hypostome but were especially abundant around the mouth and in association with the proximal and distal nerve rings (Figs. 5a–a'', d, 6b). Surrounding the mouth, the neurites of the densely packed cells plunged deeply into the epithelium. In some cases, the neurites of two neighboring type B cells appeared to intertwine (Fig. 6d). The neurites of some type B

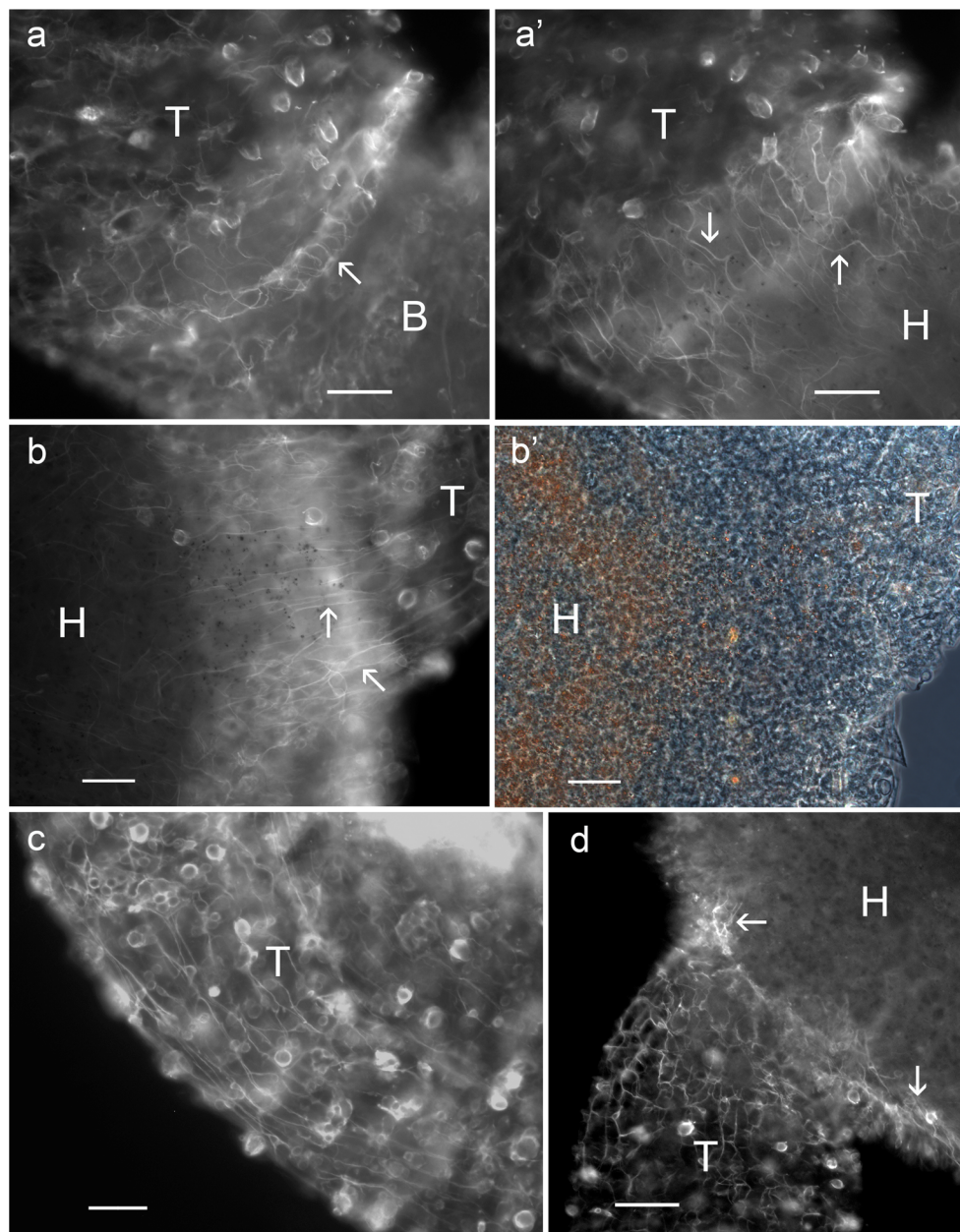


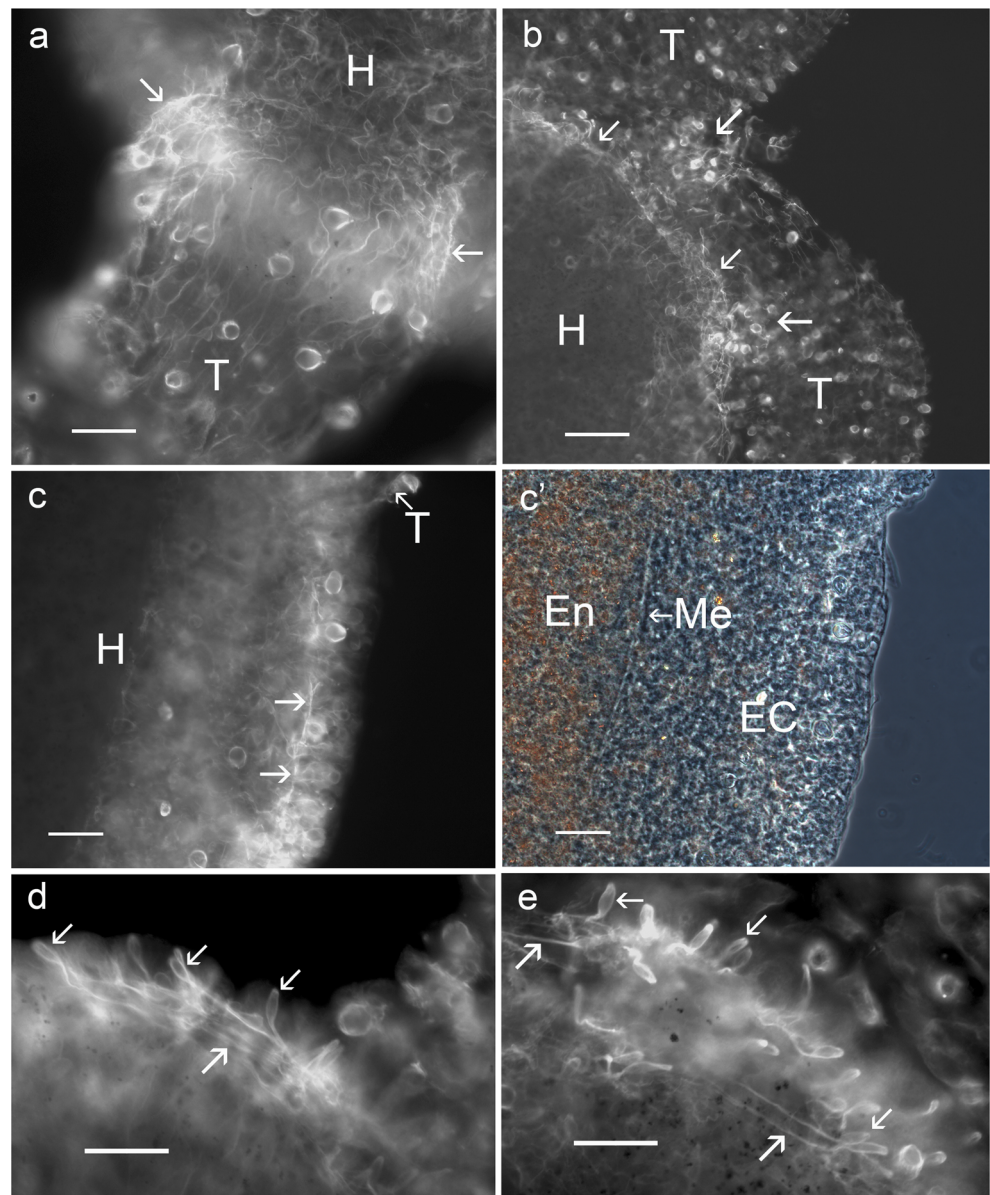
Fig. 3 **a** Sub-tentacular and supra-tentacular nerve fibers at the tentacle insertions, labeled with anti- α -tubulin antibodies. Fluorescence image of the sub-tentacular region. Note that the arrangement of fibers imparts a ladder-like appearance (*arrow*) to the proximal nerve ring (*T* tentacle, *B* body column). *Bar* 35 μ m. **a'** Fluorescence image of the supra-tentacular nerve fibers (*arrows*) that connect the tentacle and hypostomal nerve nets. Same region (but different focal plane) as in **a** (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **b** Ectodermal nerve fiber organization at the dorsal surface of the tentacle-hypostome junction. Prominent nerve fibers (*arrows*) run parallel to the axis of the tentacle, joining the nerve net of the

hypostome with that of the tentacle. Note that the proximal nerve ring is out of focus. Labeled with anti- α -tubulin antibodies (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **b'** Phase contrast image corresponding to **b** (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **c** Ectodermal nerve net of the proximal region of a tentacle, labeled with anti- α -tubulin antibodies. Note prominent longitudinal, ectodermal nerve fibers, most of which impinge on stenotele-containing cnidocytes (*T* tentacle). *Bar* = 35 μ m. **d** Regions of tangled neurites (*arrows*) at the lateral tentacle margins that are part of the proximal nerve ring. Labeled with anti- α -tubulin antibodies (*T* tentacle, *H* hypostome). *Bar* 57 μ m

cells immediately entered the distal nerve ring, causing their cell bodies to appear as a loose necklace encircling the hypostome (Fig. 6b). Type A and B cells were similarly associated with the proximal nerve ring. The neurites of several type A and B cells appeared to bifurcate close to their origins at the cell body (Fig. 6c).

The third type of putative sensory cell, type C, a tripolar cell with a pyramidal cell body, was distributed throughout the hypostome. Type C cells were seen less frequently than types A and B. In such cells, two neurites emanated from the inferior angles of the pyramid, in opposite directions (Fig. 6e''). In some of these cells, one of the neurites was directed toward the mouth, whereas the

Fig. 4 **a** Clusters of nerve cell bodies and tangled neurites (*arrows*) at the sides of a tentacle-hypostome junction. Labeled with anti- α -tubulin antibodies (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **b** Clusters of stenotele cnidocysts and tangled nerve fibers (*large arrows*) associated with fiber tangles of the proximal nerve ring (*small arrows*). Labeled with anti- α -tubulin antibodies (*T* tentacle, *H* hypostome). *Bar* 70 μ m. **c** Intertentacular portion of the proximal nerve ring (*arrows*). Labeled with anti- α -tubulin antibodies. Note several stenotele cnidocytes associated with the ring. Fluorescence image (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **c'** Phase contrast image corresponding to **c** (*En* endoderm, *EC* ectoderm, *Me* mesoglea). *Bar* 35 μ m. **d** Monopolar sensory cell bodies (*small arrows*) contribute neurites to the proximal nerve ring (*large arrow*). Labeled with anti- α -tubulin antibodies. *Bar* 35 μ m. **e** Another portion of the proximal ring, showing numerous sensory cell bodies (*small arrows*) whose fibers contribute to the ring (*large arrows*). Labeled with anti- α -tubulin antibodies. *Bar* 28 μ m



other was oriented toward the tentacles. The third angle of the cell body, namely the apex of the type C cell, typically labeled strongly with anti- α -tubulin antibodies (Fig. 6e”).

Ganglion cells of hypostome Putative ganglion cells of the ectoderm were abundantly distributed over the entire hypostome, except at the mouth (Fig. 7a–d). The neurites of these cells formed an extensive network and frequently joined both the distal and proximal nerve rings.

Hypostomes labeled with anti-GABA_B receptor antibody Genes coding for three different GABA_B R1 subunit proteins were found by inspection of the published genome of *H. vulgaris*. Initially, we aligned the immunogen with the subunit-one-like protein, XP_012556198 (which had an expected value of 3e-55; Fig. S1). Several regions of homology occurred

between the immunogen and the predicted protein, separated by amino acid sequences not represented in the immunogen (gaps). The longest region of homology contained ten amino acids (amino acids 289–298 of the hydra protein). We also used EMBOSS Matcher to perform pair-wise alignments of the immunogen with all three GABA_B R1 subunit-like proteins of *H. vulgaris* (Fig. S2). The most extensive homology was between the immunogen and the subunit-two-like protein, XP_012555926 (which had an expected value of 4e-72). A 15-amino-acid-long region of homology was found (amino acids 782–796 in the predicted protein of hydra). Shorter regions of homology in the two subunit-one-like proteins were also found.

Anti-GABA_B receptor antibody clearly labeled a ring-shaped structure that appeared to be coincident with, but not identical to the proximal nerve ring (Fig. 8a–a”). Antibody labeling of the ring was abundant and continuous. In places, the ring appeared to

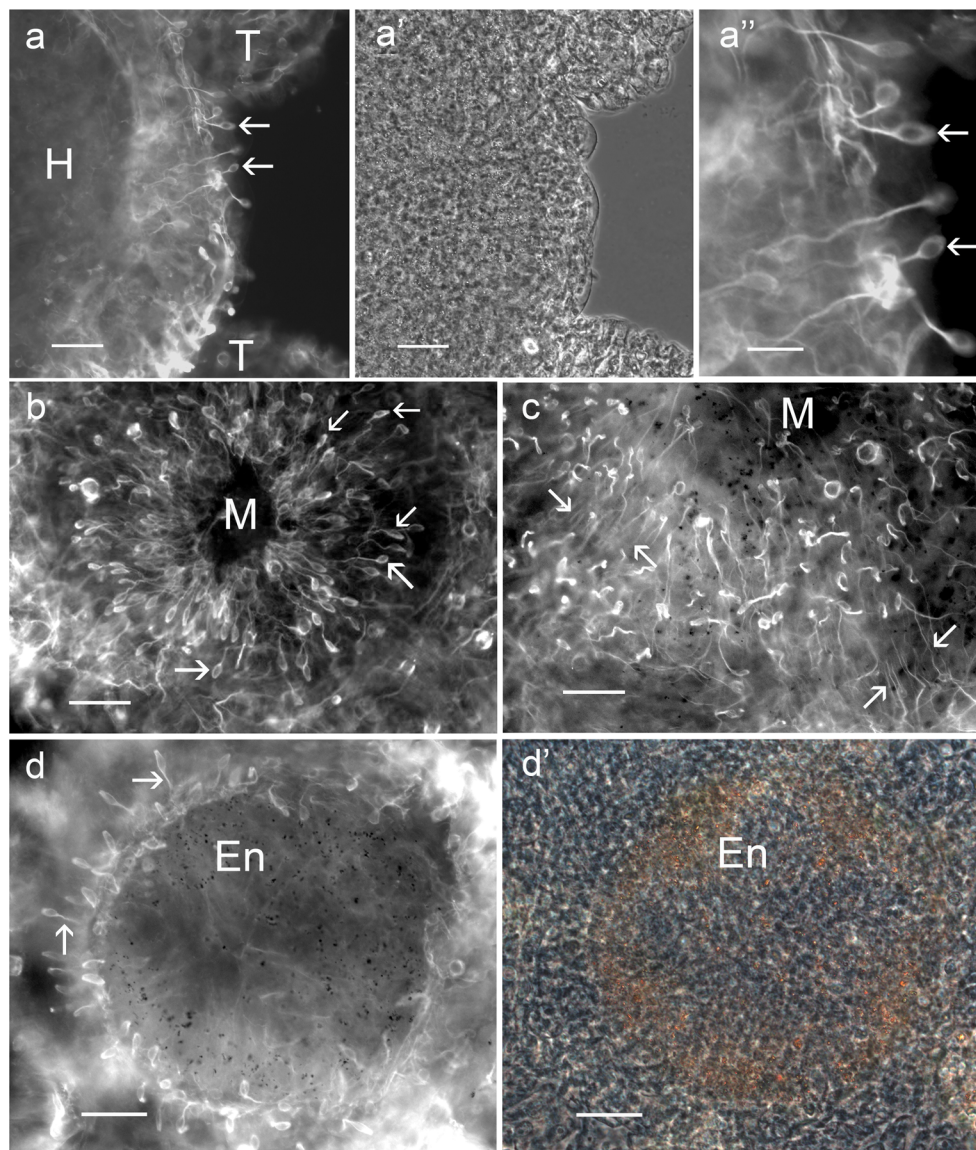


Fig. 5 **a** Portion of the proximal nerve ring between two tentacles, showing the neurites and cell bodies that contribute to the ring. Anti- α -tubulin antibodies. Fluorescence image showing sensory cell bodies (*arrows*) at the ectodermal surface (*T* tentacle, *H* hypostome). *Bar* 35 μ m. **a'** Phase contrast image corresponding to **a**. *Bar* 35 μ m. **a''** Higher magnification of **a**, showing sensory cell axons extending into the nerve ring. *Arrows* indicate sensory cell bodies. *Bar* 24 μ m. **b** Hypostome showing numerous oral sensory cells, some of which appear to be elongated ovals (*small arrows*), surrounding the mouth, whereas others are more paddle-shaped (*large arrows*). Note that some of the cell bodies

face away from the mouth opening. Enlargement of Fig. 1b'. Anti- α -tubulin antibodies (*M* mouth). *Bar* 35 μ m. **c** Sensory cells at the apex of a hypostome. The neurites of these cells extend radially down the hypostome, away from the mouth (*arrows*). Note the variety and density of tubulin-rich sensory cell bodies and neurites. Anti- α -tubulin antibodies (*M* mouth). *Bar* 35 μ m. **d** Sensory cells of the distal nerve ring. Note the short neurites (*arrows*) extending into the ring (*En* hypostomal endoderm). *Bar* 35 μ m. **d'** Phase contrast image corresponding to **d** (*En* hypostomal endoderm). *Bar* 35 μ m

be composed of more than a single fiber (Fig. 8a''); noticeable labeled nodes were apparent on either side of most tentacle insertions (Fig. 8a, a''). At higher magnification, the labeling of the proximal ring appeared as a series of discrete patches that formed a distinct ectodermal tract (Fig. 8b–b''). The GABA_B receptor labeling appeared to be confined to a narrow band, whereas the anti-tubulin-labeled fibers of the proximal nerve ring were more broadly distributed (Fig. 8a, a'', b, b'). Thus, when the anti-tubulin labeling was compared with the anti-

GABA_B receptor labeling, the anti-tubulin labeling was clearly seen on the extensively branched elements of the proximal nerve ring, whereas the GABA_B receptor labeling was restricted to numerous discrete and compact loci. We did not observe anti-GABA_B receptor labeling coincident with the distal nerve ring, indicating that the labeling was selective.

Patches of GABA_B receptor labeling also occurred on stenotele cnidocytes (Fig. 8c–c''). The GABA_B receptor label was restricted to small loci near the base of the cnidocyst,

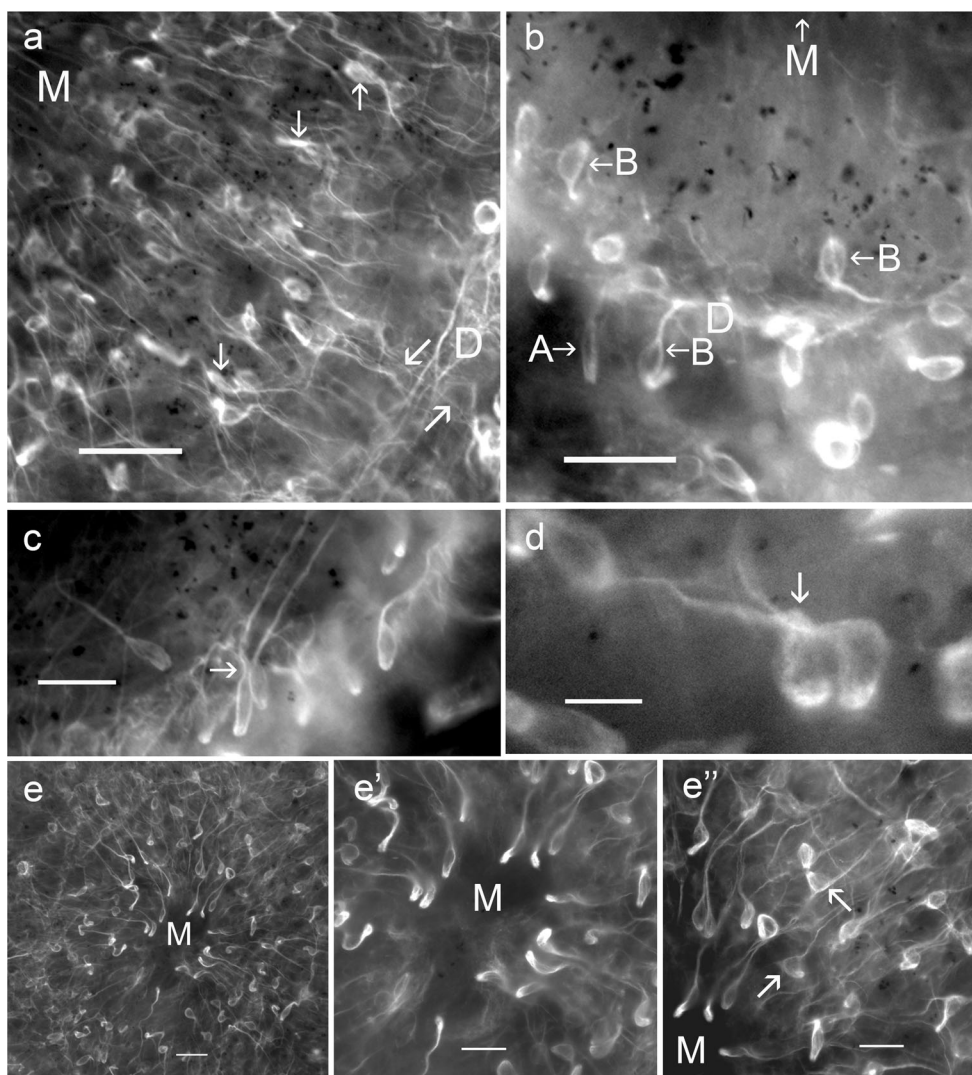


Fig. 6 **a** Two types of sensory cells at the mouth opening of the hypostome. *Small arrows* indicate types A and B sensory cells visible at high magnification. Neurites of the sensory cells (*large arrows*) are directed away from the mouth opening and extend beyond a portion of the distal nerve ring. Anti- α -tubulin antibodies (*D* distal nerve ring, *M* mouth). *Bar* 30 μ m. **b** Single axons emanating from cell bodies of type A and B cells join the distal nerve ring. Anti- α -tubulin antibodies (*A* type A cell, *B* type B cell, *M* mouth, *D* distal nerve ring). *Bar* 20 μ m. **c** Neurites from the cell bodies of two type A cells appear to cross (*arrow*). Anti- α -tubulin antibodies. *Bar* 18 μ m. **d** Neurites of two type

B cells appear to be intertwined (*arrow*). Anti- α -tubulin antibodies. *Bar* 18 μ m. **e** Sensory cells of the hypostome. The cell bodies of elongated sensory type A cells closely appose the opening of the mouth at the apex of the hypostome. Anti- α -tubulin antibodies (*M* mouth). *Bar* 22 μ m. **e'** Enlargement of a portion of **e**, showing many type A sensory cells. Anti- α -tubulin antibodies (*M* mouth). *Bar* 14 μ m. **e''** Enlargement of a portion of **e**, showing the cell bodies of type C pyramidal cells (*arrows*) located near the mouth opening. Anti- α -tubulin antibodies (*M* mouth). *Bar* 14 μ m

above the putative cnidocyte nucleus (Fig. 8c). In contrast, anti-tubulin labeling of the cnidocyte was localized laterally in the cnidocyst, presumably coincident with the microtubular scaffold surrounding the cnidocyst (Fig. 8c').

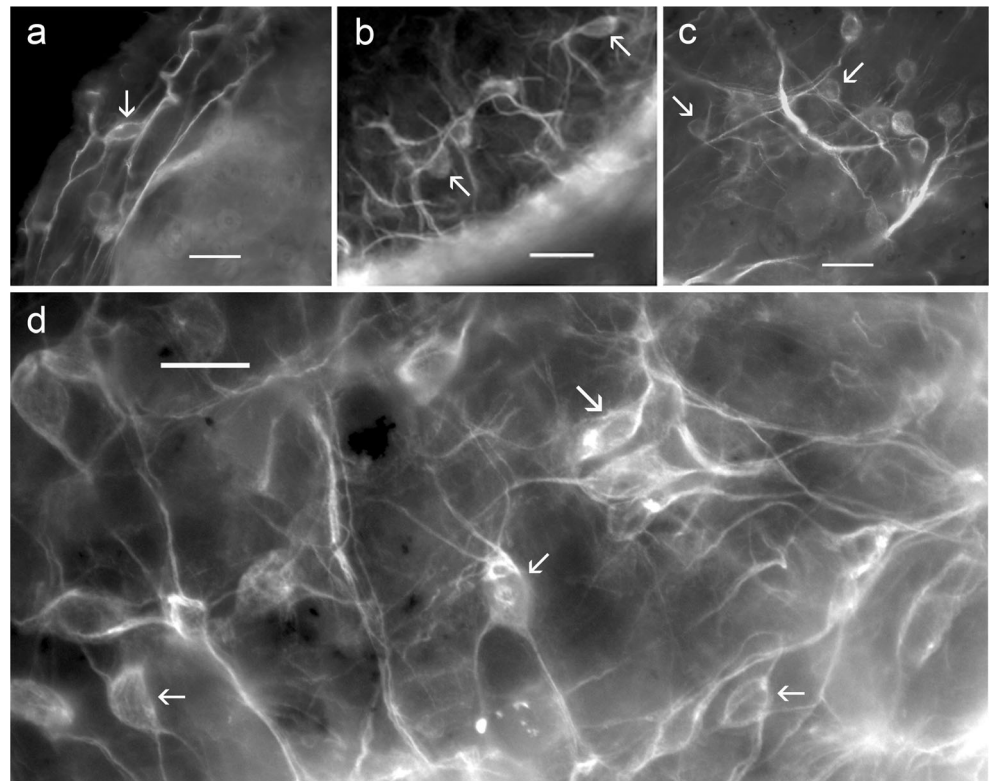
In the apical region of the hypostome, extensive anti-GABA_B receptor labeling was found to coincide with the orange pigmentation characteristic of the endodermal tissue within the folds surrounding the oral cavity but was diminished or absent in the tissue at the edges of the folds, closest to the cavity (Fig. 8a, a''). Some of the labeling was discrete and punctate, whereas other labeling was more diffuse (Fig. 8a'').

We were unable to identify the types of endodermal cells that were labeled. However, the label was broadly distributed throughout the endoderm.

Discussion

Our studies with anti- α -tubulin antibodies have revealed the presence of at least two ectodermal nerve rings in the hypostome of *Hydra vulgaris*: a proximal nerve ring at the level of the tentacle insertions (comprising parallel and anastomosing

Fig. 7 a–c Anastomosing nerve fibers in the hypostome in the vicinity of the proximal nerve ring. Neurites and cell bodies (arrows) of ganglion cells contribute to the nerve ring. Anti- α -tubulin antibodies. Bars 14 μ m (a, c), 20 μ m (b). **d** Hypostomal ganglion cells in the vicinity of the proximal nerve ring. Numerous bipolar and multipolar ganglion cells (small arrows) interconnect in the ectodermal hypostomal nerve net. A cluster of cell bodies is indicated by a large arrow. Anti- α -tubulin antibodies. Bar 11 μ m



nerve fibers) and a structurally similar distal nerve ring midway between the mouth and the ring of tentacles in the hypostomal dome. Distributed throughout the hypostomal ectoderm are at least three morphologically distinct types of sensory cells and many ganglion cells. In addition, we found extensive anti-GABA_B receptor antibody labeling distributed in a ring coincident with the proximal nerve ring. However, a similar ring, coincident with the distal nerve ring was not observed. Abundant, broadly distributed, GABA_B receptor antibody labeling was detected in the hypostomal endoderm.

Oral cavity Based on our examination of many hypostomal preparations, the mouth cavity appears to be formed by several epithelial folds of mainly endodermal tissue. In phase contrast images of those preparations in which the mouth was clearly closed, the ectodermal epithelial tissue appeared to form a continuous layer over the mouth. These observations are consistent with those of Beams et al. (1973), Westfall and Townsend (1976), Wood (1979a, 1979b), Campbell (1987), and Technau and Holstein (1995). In fluorescent images of animals labeled with anti- α -tubulin or anti-GABA_B receptor antibodies, a central dark star-shaped region was evident that was presumably attributable to the lack of labeling in this region. The presence of a star pattern supports earlier electron microscopic evidence that the cavity of the mouth is surrounded by highly folded epithelial tissue (Wood 1979a). Thus, the folds permit the mouth to expand, without tearing, to accommodate large prey objects. In an immunocytochemical

study of *H. vulgaris*, Technau and Holstein (1995) have described highly expandable endodermal epithelial cells at the boundary between the ectoderm and endoderm, at the apex of the mouth, which allow the mouth to open without rupturing. These cells are presumably those that were previously described by Wood (1979a, 1979b) and Campbell (1987) in electron microscopic (transmission and scanning) studies. Our study does not provide information about these cells.

Proximal nerve ring This study presents the first published, morphological evidence for a ring of neurites and cell bodies at the level of the tentacles in *H. vulgaris*. The fibers of the ring emanate from and extend into the tentacles and hypostome. The neurites and cell bodies of the ring coalesce into prominent aggregates at the lateral margins of the tentacle insertions. These may be related to clusters of ganglion cells identified near tentacle insertions in serial section reconstructions from electron micrographs of *H. littoralis* (Kinnamon and Westfall 1981a) and, more recently, as opsin-positive nodes identified in in situ-labeled *H. magnipapillata* (Plachetzki et al. 2012).

Distal nerve ring In addition to the proximal nerve ring, we also present the first description, in *H. vulgaris*, of circumferentially running nerve fibers that appear to make up a distinct but loosely organized distal nerve ring. The cells, whose neurites contribute to the ring, are mainly sensory cells, although ganglion cell neurites may also contribute.

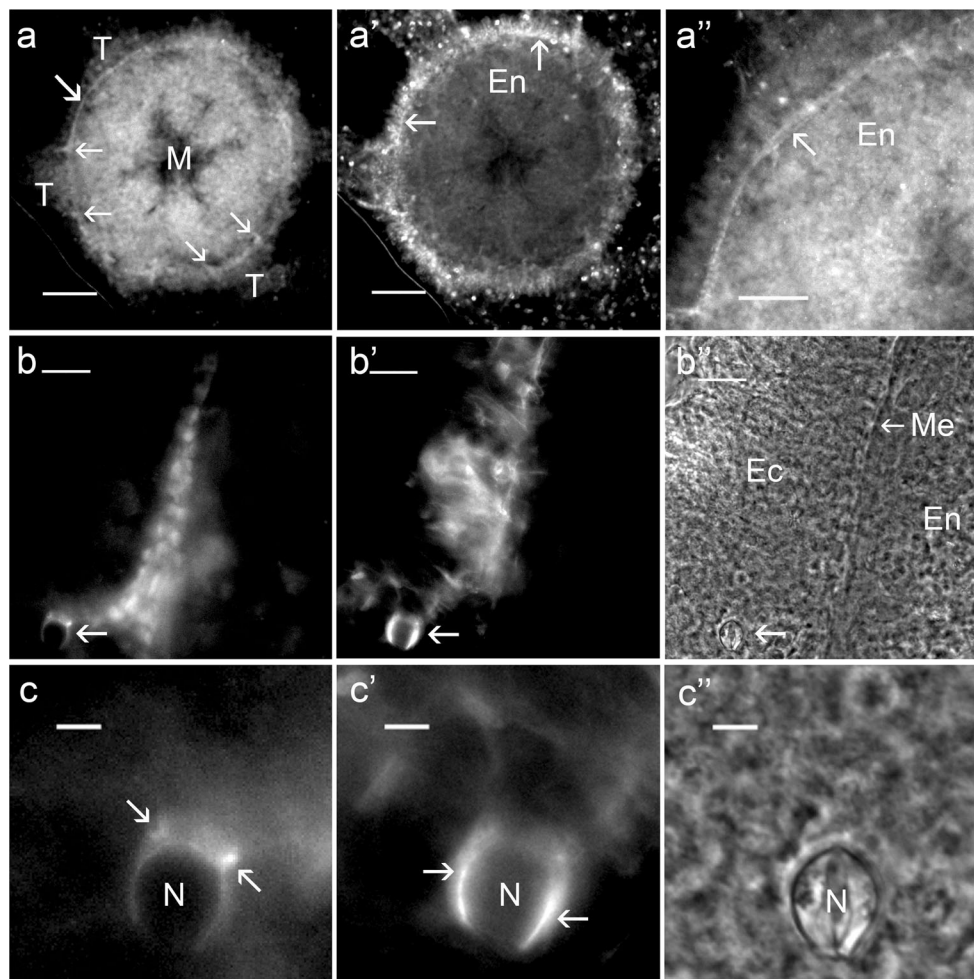


Fig. 8 **a–a''** *En face* views of an entire hypostome, double-labeled with anti- α -tubulin and anti-GABA_B receptor (GABA_BR) antibodies. The proximal nerve ring (**a'**, *arrows*) and the GABA_BR ring (**a**, *large arrow*) are coincident. **a** The anti-GABA_BR ring appears to have nodes (*small arrows*), most of which are located on either side of the tentacle insertions (*T* tentacle, *M* mouth). Bar 11 μ m. **a'** Anti- α -tubulin image in the same focal plane as **a** (*En* endoderm). Bar 11 μ m. **a''** Higher magnification of **a**. The GABA_BR ring appears to comprise parallel strands (*arrow*). Note the extensive granular GABA_BR labeling of the hypostomal endoderm in **a**, **a''** and the paucity of labeling of the endoderm with anti- α -tubulin antibody in **a'** (*En* endoderm). Bar 56 μ m. **b–b''** High magnification views of a portion of the hypostome shown in **a**, **a'** but double-labeled with anti- α -tubulin and anti-GABA_BR antibodies, near the insertion of a tentacle emanating from the hypostome. **b** Anti-GABA_BR. **b'** Anti- α -tubulin antibody. **b''**

Phase contrast. As seen in **b''**, the boundary between the ectoderm and endoderm is marked by the mesoglea. Note the differences in the pronounced patchiness of the anti-GABA_BR labeling and the more fibrous labeling by the anti- α -tubulin antibody. Note also that the anti- α -tubulin antibody-labeled fibers of the nerve ring branch out into the hypostomal ectoderm, whereas similar branching is not apparent following GABA_BR labeling. A stenotele cnidocyte is indicated by *arrows* (*Ec* ectoderm, *En* endoderm, *Me* mesoglea). Bars 14 μ m. **c–c''** Enlargements of portions of **b–b''**, showing anti- α -tubulin and anti-GABA_BR labeling associated with a stenotele cnidocyte (facing *downward*). Labeling with anti-GABA_BR antibodies is localized at the base of the cnidocyst (*arrows*). **c'** Bright labeling with anti- α -tubulin antibody indicates the location of a microtubular scaffold surrounding the cnidocyst (*arrows*). **c''** Corresponding phase contrast (*N* cnidocyst). Bars 3.4 μ m

These findings correlate with our earlier electrophysiological studies suggesting that the ectodermal pacemaker system exists at or near the bases of the tentacles (Kass-Simon 1972, 1973; Kass-Simon and Passano 1978). The pacemaker system is responsible for the periodic ectodermal body-column contractions (Passano and McCullough 1964). It was originally considered to be composed of a number of circumferentially connected nerve cells that reciprocally interact with each other and with the tentacle pacemaker systems (Rushforth and Burke 1971; Kass-Simon 1972, 1973; Kass-Simon and

Passano 1978). The electrophysiological data indicated that the ectodermal pacemaker system actually comprises a set of several interconnected neuronal loci that are situated at or near the bases of the tentacle insertions whose activity is conducted circumferentially around the hypostome.

Body contraction impulses are induced as a result of increasing activity in the tentacles (Kass-Simon 1972, 1973). Facilitated excitation in the hypostomal pacemaker loci produce concerted contractions of all the tentacles and the body column (Kass-Simon 1972). Our present findings describing

the proximal nerve ring, with its ostensible neuropil on either side of the tentacle insertions and its relationship to the tentacular nerve net, seem to provide the morphological basis for the behavioral physiology.

Relationship of *H. vulgaris* nerve rings to those of other cnidaria The location of the *H. vulgaris* distal nerve ring appears to be similar to that of the densely organized rings that have been found in *H. oligactis* and other hydra species and that label with anti-RFamide, anti-FMRFamide, and other peptide antibodies or with JD1 or RC9 monoclonal antibodies (Dunne et al. 1985; Grimmelikhuijzen 1985; Yu et al. 1985; Koizumi et al. 1992). Although these previous studies failed to find an anti-RFamide antibody-labeled hypostomal nerve ring in *H. vulgaris*, our recent studies indicate that both the proximal and distal nerve rings label with anti-RFamide antibody (Munro 2014). Whether either of these rings in *H. vulgaris* is homologous to the single hypostomal rings in other species is still unclear. Our studies and those cited here clearly reveal that different antibodies or protocols give dramatically different impressions of the hydra nervous system (Cristino et al. 2007).

A nerve ring at the location of the proximal nerve ring of *H. vulgaris* has not previously been described in immunohistochemical studies of other species of hydra, although its counterpart may have been encountered in ultrastructural studies of *H. viridis* and *Pelmatohydra* (Davis et al. 1968; Matsuno and Kageyama 1984), and in situ hybridization studies in *H. magnipapillata* (Plachetzki et al. 2012). Therefore, the question arises whether hydras, other than *H. vulgaris*, also have more than one hypostomal nerve ring.

In a thought-provoking review article, Koizumi (2007) has suggested that the peptide-labeled hypostomal nerve rings of stalked hydras (*H. oligactis*, *H. robusta*, *H. pseudoligactis*) and gracile hydras (*H. utahensis*, *H. circumcincta*, *H. hymanae*) reflect the origin of the bilaterian central nervous system in an ancestral cnidarian. Indeed, the idea that cnidarian nerve rings are ancestral to the neuronal centralization in higher animals has been expressed in a number of publications (Watanabe et al. 2009; Koizumi et al. 2015). In conjunction with the behavioral electrophysiology, our present observation of at least two nerve rings in *H. vulgaris* gives weight to the idea that the nervous system of the hypostome represents a central coordinating and integrating system.

Koizumi et al. (1992) have suggested that the peptide-positive ring is involved in the control of both feeding and the contraction behavior of hydra, whereas, in *H. vulgaris*, our observation of two hypostomal nerve rings suggests that they mediate different behavioral responses. Thus, the distal ring, which receives input from the sensory cells surrounding the mouth, might be the ring that is primarily responsible for the control of the mouth movements during feeding, whereas the proximal ring, which connects the bases of the tentacles

and links the hypostomal, tentacular, and body nerve fibers, is mainly responsible for their interactions and the control of tentacle and body contractions. Nonetheless, neurites of the cells of the two rings interconnect with each other, and during feeding, body contractions are inhibited (Rushforth and Hofman 1972), supporting the idea that the two rings have disparate, but interacting functions. Our recent electrophysiological experiments (B.M. Lauro, V. Nandivada and G. Kass-Simon, unpublished) indicate that reduced glutathione (GSH), which elicits hypostomal feeding behavior, also inhibits proximal pacemaker activity.

The existence of two rings in *H. vulgaris* is consistent with the body plans of other hydrozoans. In hydrozoan medusae, two ectodermal nerve rings coordinate tentacle and bell activity (Singla 1978a, 1978b; Roberts and Mackie 1980; Satterlie and Spencer 1983). As pointed out by Koizumi et al. (1992), in *Polyorchis penicillatus*, the motor neurons of the inner nerve ring coordinate the swimming muscles (Satterlie and Spencer 1983), whereas the outer ring integrates sensory information (Spencer and Arkett 1984). The distal ring of *H. vulgaris* may be functionally analogous to the medusan outer ring, whereas the proximal ring may be analogous to the inner ring.

Sensory nerve network Ectodermal sensory cells were distinguished on the basis of their characteristic morphologies and locations (see **Materials and Methods**). Our images indicate the presence of at least three types of morphologically differentiated sensory cells: those with thin fusiform bodies (type A), those with broader club-shaped bodies (type B), and those with pyramidal cell bodies (type C). Types A and B were earlier described by Hadzi (1909), whereas types C and A were described by Yu et al. (1986). In previous studies of the tentacles of hydra, cells similar to the hypostomal type C cells were labeled with RC14, an antibody specific for sensory cells of the tentacle (Erskine 1989). In both the tentacle and the hypostome, the intense anti- α -tubulin labeling at the apex of the cells suggests the presence of a sensory cilium, although our images do not clearly reveal cilia, undoubtedly because they are recessed within the sensory cell cone of stereocilia (Kinnamon and Westfall 1984).

The existence of the three morphologically different cell types suggest that several types of sensory modalities might be perceived by the hypostome. Electrophysiological and behavioral evidence exists for the chemoreceptive response of the hypostomal ectoderm to GSH (Loomis 1955; Lenhoff 1961; Bellis et al. 1992; Pierobon et al. 1995; Grosvenor et al. 1996; Lauro 2015). Another sensory modality likely to be transduced in the hypostome is mechanoreception, since lightly touching the hypostome of hydra results in body column and tentacle contractions (G. Kass-Simon, unpublished observations). Well-documented photic responses and extensive opsin labeling in the hypostome suggest that some

of the sensory cells are electromagnetic (light) receptors (Plachetzki et al. 2012; Passano and McCullough 1962, 1964, 1965; Taddei-Ferretti and Musio 2000; Guertin and Kass-Simon 2015). The organization of the putative hypostomal sensory nerve net is consistent with the idea that these cells are involved in the feeding behavior of *H. vulgaris* (Wood 1979a, 1979b; Kinnamon and Westfall 1981a, 1981b, 1984; Yu et al. 1985, 1986). The cone of the hypostome is densely carpeted with putative sensory cells, many of which surround the mouth, whereas others are more closely associated with the nerve rings. Thus, the neurites of all the sensory cells, together with the neurites of the putative hypostomal ganglion cells, form an integrated neuronal network that appears to couple sensory information to the effector systems of the hypostome.

GABA_B receptors Using polyclonal antibodies prepared against mammalian GABA_B R1, we have determined localizations of label that suggest the presence of GABA_B receptors in the hypostome of *H. vulgaris*. These findings are supported by genomic evidence for GABA_B receptors in hydra (www.ncbi.nlm.nih.gov) and with genomic evidence that GABA_B receptor proteins occur in other Cnidaria (Ancil 2009).

The distribution of our observed labeling is consistent with electrophysiological and behavioral evidence for the localized roles of GABA_B receptors in the hypostome. Biochemical, behavioral, and electrophysiological data indicate that GABA affects behavioral and pacemaker systems in hydra (Pierobon et al. 1995; Kass-Simon et al. 2003), and that GABA_B ligands specifically affect the contraction-burst pacemaker system (Lauro 2015).

The conspicuous presence of putative GABA_B receptors in the oral endoderm is consistent with our published findings that GABA reduces the frequency of endodermal rhythmic potential pacemaker pulses (Kass-Simon et al. 2003) and with the observation that the duration of GSH-induced mouth opening is prolonged by the application of GABA (Pierobon et al. 1995). Further, our recent electrophysiological studies indicate that GABA_B agonists affect GSH-induced hypostomal impulses recorded at the mouth (B.M. Lauro, V. Nandivada and G. Kass-Simon, unpublished). The extensive distribution of putative GABA_B receptors associated with the proximal ectodermal nerve ring is also consistent with the idea that GABA is involved in modulating the output of the hypostomal contraction burst system. The application of GABA decreases the frequency of contraction bursts and the number of pulses/burst (Kass-Simon et al. 2003). Furthermore, GABA_B ligands decrease the frequency of GSH-induced electrical activity in the hypostome (Lauro 2015). The participation of GABA_B receptors in the neuroeffector systems of hydra is further evidenced by the involvement

of GABA_B receptors in cnidocyst discharge in the tentacles (Kass-Simon and Scappaticci 2004; Scappaticci and Kass-Simon 2008).

Concluding remarks In summary, we suggest that *H. vulgaris*, and perhaps other species of hydra, have at least two hypostomal ectodermal nerve rings that are functionally distinct, but nevertheless coordinated. The proximal nerve ring, associated with GABAergic neurotransmission, may be primarily responsible for pacemaker functions, whereas the distal nerve ring might directly control the hypostomal feeding behavior. Further, the presence of at least three types of sensory cells that contribute to both rings suggests that various types of stimuli are perceived and integrated in the hypostome.

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