Novel, posterior sensory organ in the trochophore larva of *Phyllodoce maculata* (Polychaeta)

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A new posterior sensory organ (PSO), located at the dorsal midline of the hyposphere, is described by immunocytochemical detection of acetylated α -tubulin and serotonin (5-HT) in a laser-scanning microscope, as well as three-dimensional reconstructions after optical serial sectioning in the trochophore larva of the polychaete *Phyllodoce maculata* (Phyllodocidae). The unpaired PSO consists of five bipolar sensory cells, two of them being 5-HT immunopositive, which send axons to the cerebral ganglion and prototroch nerve. The dendrites of these cells project to the surface and bear one cilium each. A single neuronal fibre from the apical sensory organ innervates the PSO.

Keywords: Annelida; nervous system; serotonin; acetylated tubulin

1. INTRODUCTION

Regarded as a common ancestral form for all Trochozoa, trochophores have long been a focus of comparative studies in anatomy, systematics and evolution (see Nielsen 2001). Despite their microscopic size and relatively simple structure, trochophores display a considerable repertoire of complex behavioural action patterns that are mediated by a centralized nervous system and peripheral sensory receptors (Kempf *et al.* 1997). Studies on neuronal development in larval Trochozoa have shown that they possess a well-developed nervous system, which generally includes several centres: cerebral ganglia, ventral cords and prototroch nerve (Åkesson 1967; Lacalli 1981, 1984, 1986; Barlow & Truman 1992; Kempf *et al.* 1992; Hay-Schmidt 1995; Dickinson *et al.* 2000; Voronezhskaya *et al.* 2002, 2003). This system obviously has to detect and process incoming environmental stimuli. Meanwhile, the only well-described trochophore sensory structure is the apical sensory organ (Conklin 1897; Lacalli 1981, 1984; Kempf *et al.* 1997; Page 2002).

Until recently, detailed neuroanatomical studies on invertebrate larvae were limited by their small size and nervous structures, being sometimes beyond the resolution of optical microscopy. Transmission electron microscopy can sample only small parts of a larva, so key concentrations of neurons can be missed if their location is not already known (Lacalli & Gilmour 2001). Modern

confocal laser-scanning microscopy (CLSM), in combination with highly sensitive and selective neuronal markers and bright, photo-stable fluorescent probes, solve the problem and allow the study of the whole nervous system at the level of single neurons.

We used the advantages of CLSM for the detailed study of neurogenesis in various trochozoan animals (Voronezhskaya *et al.* 1999, 2002, 2003), and, in the course of a study on larval neurogenesis, we encountered a sensory organ in the dorso-posterior region of the polychaete *Phyllodoce maculata* that, as far as we are aware, was previously unknown. This organ is described herein in detail by means of immunochemical detection of serotonin (5-HT) and acetylated α -tubulin combined with CLSM, as well as standard light microscopic reconstruction of serial sections.

2. MATERIAL AND METHODS

Adult specimens of *P. maculata* L., 1767 were collected in the Chupa Bay, White Sea (Marine Biological Station of the St Petersburg University) and kept in pairs in aerated aquaria. After spawning, egg cocoons were kept in Petri dishes in filtered sea water (FSW) at 14–15 °C. Hatchlings were kept in FSW (one to two animals per millilitre). Trochophores were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (PB; pH 7.4) for 4 h at 14–15 °C, washed in PB, transferred into 30%, 50% and 70% ethanol and stored at –20 °C. For immunocytochemistry, larvae were washed in PB (3 × 20 min) and incubated in a mixture of anti-5-HT (DiaSorin, USA, no. 20091, polyclonal, rabbit, diluted 1 : 2000) and anti-acetylated α -tubulin (Sigma, USA, T-6793, monoclonal, mouse, 1 : 1000) primary antibodies (ABs) in PB with 10% normal goat serum, 0.25% bovine serum albumin, 1% Triton X-100 (TX) and 0.03% sodium azide for 3 days at 10 °C. The specimens were washed in PB, incubated in a mixture of goat anti-rabbit Alexa 488 immunoglobulins (IgG) and goat anti-mouse Alexa 546 IgG (Molecular Probes, USA, A-11008 and A-11003), both diluted 1 : 800 in PB-TX, overnight at 10 °C. The specimens were washed in PB, immersed in 60% glycerol in PB and mounted on glass slides. If necessary, 5 μ g ml⁻¹ of propidium iodide (PI, P4170, Sigma, USA) was added to the mounting medium to label cell nuclei.

The specimens were examined with an LSM-510 laser-scanning microscope (Carl Zeiss, Germany) with appropriate wavelength-filter configuration settings (100 embryos at each developmental stage). Three-dimensional, rotatable reconstructions were produced by using LSM-510 software. For illustrations, maximum-intensity projections of optical sections were prepared. Controls included replacement of the primary ABs with non-immune serum and pre-incubation of anti-5-HT ABs overnight with 5-HT-bovine serum albumin (BSA) conjugate (Voronezhskaya *et al.* 2002). No specific staining was observed in control preparations. Reversal of the fluors conjugated to secondary ABs gave identical results.

For serial sectioning, the specimens were dehydrated through an ethanol series followed by propylene oxide, embedded in Poly/Bed 812 (Polysciences, USA), sectioned at 2 μ m with an LKB-4 ultratome (LKB, Sweden) using glass knives, stained with methylene-blue and then examined and photographed with an Axiovert 200 microscope equipped with a digital camera. Four complete series of hatchlings were analysed.

3. RESULTS

An unpaired posterior sensory organ (PSO) was constantly detected at the dorsal midline of the hyposphere in trochophores of *P. maculata* starting from hatching (figure 1a,b). It comprised five bipolar sensory cells, two of which were 5-HT immunoreactive (IR) and sent basal fibres to the circular nerve running under the epithelium of the prototroch (prototroch nerve). Each of the five cell bodies (determined by propidium iodide DNA staining) had a single apical dendrite, which projected to the surface and bore a single cilium (figure 1b–d). Thus, the surface tuft always contained five cilia. The axonal processes of the two 5-HT IR cells (consistently a branched axon of cell 1 and two independent axons of cell 2) entered the prototroch nerve, and the fibres of each cell ran in both direc-

Figure 1. (Caption overleaf.)

tions (figure 1e). In addition, tubulin immunoreactivity revealed three more 5-HT immunonegative fibres, which connected the PSO to other parts of the nervous system (figure 1f). One fibre arrived from the apical organ,

entered the PSO and terminated without a cilium (figure 1b,d,f, filled arrowheads). Two 5-HT immunonegative axons of the PSO cells ran to the right cerebral ganglion (figure 1b,f, open arrows).

Figure 1. Posterior sensory organ in the newly hatched trochophore, anti-acetylated α -tubulin (red—*a, b, d–h*) and anti-5-HT (green—*a, b, d–h*) immunolabelling, and PI labelling of nuclei (*c*). For three-dimensional rotatable reconstructions see electronic Appendices A–C, available on The Royal Society's Publications Web site). (*a*) Latero-caudal view. The unpaired sensory organ is located on the dorsal side of the hyposphere (arrow); mo, mouth opening; pn, prototroch nerve; pt, prototroch (number and step size of optical sections: $66 \times 0.8 \mu\text{m}$). (*b*) Dorsal view on the PSO showing two 5-HT IR cells (1 and 2), the fibre arriving from the episphere (filled arrowheads) and two axons emanating from the cells of the PSO (open arrows); pn, prototroch nerve ($68 \times 0.35 \mu\text{m}$). (*c*) Nuclei of five cells in the PSO (1–5, propidium iodide staining, red), and tubulin IR dendrites (green) ($22 \times 0.8 \mu\text{m}$). (*d*) Apical part of the PSO showing monociliated dendrites (in red) ($31 \times 0.35 \mu\text{m}$). (*e*) Two 5-HT IR cells (1 and 2), each projecting two basal fibres into the prototroch nerve (pn) ($27 \times 0.75 \mu\text{m}$). (*f*) Pretrochal region of the specimen depicted in (*b*) showing the fibre (filled arrowhead), which goes to the PSO from the apical organ (ao), and two fibres (open arrows) arising from the PSO and entering the right cerebral ganglion (cg) ($68 \times 0.35 \mu\text{m}$). (*g*) Reduced PSO in the two-day-old trochophore ($20 \times 1.25 \mu\text{m}$). (*h*) Five days after hatching; the remnants of degenerating PSO ($14 \times 1.25 \mu\text{m}$). Scale bars, $10 \mu\text{m}$.

In the course of development, immunoreactivities to both 5-HT and tubulin in the PSO gradually disappeared. Two days after hatching, anti-tubulin labelling weakened and the 5-HT IR cell 2 became undetectable (figure 1*g*). Five days after hatching (late trochophore–early metatrochophore stage), the whole PSO became undetectable, and only degenerating remnants of it could be seen in 20% of the specimens (figure 1*h*). At the later stages, no remnants were found.

The structure of the PSO was further confirmed by reconstructions of serial sections. A group of five elongated neuron-like cell bodies was consistently found in this region below the epithelium (figure 2*a*) sending dendrites to the surface, and axons to the base of the prototroch (figure 2*b*).

4. DISCUSSION

The structure described above is undoubtedly a sensory organ. The set of characteristic features, bipolar cells bearing cilia and sending axonal fibres to the CNS, 5-HT immunolabelling and dense concentration of microtubules in both dendritic and axonal processes, unambiguously points to its sensory nature (figure 2*c*). None of our data suggest a photoreceptive function, so the PSO is probably chemo- or mechanosensory.

No structures similar to the PSO have previously been described in trochozoan larvae. In polychaete trochophores, only the apical organ and ventral pretracheal sensory cell are known (Åkesson 1967; Lacalli 1981, 1984, 1986; Hay-Schmidt 1995; Voronezhskaya *et al.* 2003). In chiton larvae, transient dorsolateral sensory cells were described, but these cells are pretracheal, paired and FMRFa immunopositive (Haszprunar *et al.* 2002; Voronezhskaya *et al.* 2002). Post-trochal peripheral neurons occur in larval gastropods, but they are solitary FMRFa IR cells, which appear at much earlier stages and

Figure 2. (*a*) Parasagittal section showing the PSO (arrow) in the dorsal hyposphere. (*b*) Two sensory cells (presumably 1 and 4) send dendrites (arrow) to the surface and axons (filled arrowhead) to the base of the prototroch (asterisk). Scale bars, $10 \mu\text{m}$. (*c*) Schematic drawing of the PSO, dorsal view. Five sensory cells (1–5) extend dendrites projecting to the surface and bearing one cilium each, and axons, which enter the prototroch nerve (pn) or run to the cerebral ganglion (cg). The cells 1 and 2 are 5-HT IR. A fibre from the apical organ (ao) enters the sensory structure.

have a different morphology and projection pattern (reviewed by Croll 2000). By contrast, no FMRFa immunoreactivity is detected in the PSO of *P. maculata*, while in the other parts of the trochophore, peripheral FMRFa immunopositive cells are abundant (Voronezhskaya *et al.* 2003).

Dorsal epidermal sensory structures (nuchal organs) are known in adult polychaetes (e.g. Purschke 1997; Jelsing 2002), but their location and morphology are, basically, different. These differences, together with the fact that both 5-HT and tubulin immunoreactivities in the PSO disappear during the beginning of metamorphosis, indicate that the PSO is not a rudiment of the nuchal organ but a specifically larval sensory structure.

Bipolar neurons that bear cilia, contain serotonin, innervate the main locomotory structures (velum or prototroch) and disappear after metamorphosis have been

described in the apical organ of various trochozoan larvae including *P. maculata*, and a sensory-motor function for these cells has been proposed (Lacalli 1986; Kempf *et al.* 1997; Page & Parries 2000; Kuang & Goldberg 2001; Voronezhskaya *et al.* 2002, 2003). The two 5-HT immunoreactive cells of the PSO have the same features and the same fate although they are located at the back of the trochophore. Nonetheless, the two organs are obviously connected, as indicated by the linking neuronal fibre.

Transmitter content of the other three neurons in the PSO is unknown. Two of them do not extend axons to the prototroch nerve, but instead project axons into the developing cerebral ganglion. Thus, the PSO is directly connected to the main larval locomotory structure, the prototroch, and at least two main nerve centres, the apical organ and the cerebral ganglion. The function of the PSO is, as yet, unclear; however, it may be said that the PSO is a transient larval sensory structure restricted to the planktonic phase of the life cycle of *Phyllodoce*.

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- Åkesson, B. 1967 On the nervous system of the *Lopadorhynchus* larva (Polychaeta). *Arkiv Zool.* **20**, 55–78.
- Barlow, L. A. & Truman, J. W. 1992 Patterns of serotonin and SCP immunoreactivity during metamorphosis of the nervous system of the red abalone, *Haliotis rufescens*. *J. Neurobiol.* **23**, 829–844.
- Conklin, E. G. 1897 The embryology of *Crepidula*. *J. Morphol.* **13**, 1–230.
- Croll, R. P. 2000 Insights into early molluscan neuronal development through studies of transmitter phenotypes in embryonic pond snails. *Microsc. Res. Technique* **49**, 570–578. (DOI 10.1002/1097-0029(20000615)49:6<570::AID-JEMT7>3.0.CO;2-Q.)
- Dickinson, A. J. G., Croll, R. P. & Voronezhskaya, E. E. 2000 Development of embryonic cells containing serotonin, catecholamines and FMRFamide-related peptides in *Aplysia californica*. *Biol. Bull.* **199**, 305–315.
- Haszprunar, G., Friedrich, S., Wanninger, A. & Ruthensteiner, B. 2002 Fine structure and immunochemistry of a new chemosensory system in the chiton larva (Mollusca: Polyplacophora). *J. Morphol.* **251**, 210–218. (DOI 10.1002/jmor.1084.)
- Hay-Schmidt, A. 1995 The larval nervous system of *Polygordius lacteus* Scheinder 1868 (Polygordiidae, Polychaeta): immunocytochemical data. *Acta Zool. (Stockholm)* **76**, 121–140.
- Jelsing, J. 2002 Ultrastructural investigations on the cephalic and metameric nuchal organs of *Spio* cf. *filicornis* (Polychaeta, Spionidae). *Zoomorphology* **121**, 213–220. (DOI 10.1007/s00435-002-0060-1.)
- Kempf, S. C., Chun, G. V. & Hadfield, M. G. 1992 An immunocytochemical search for potential neurotransmitter in larvae of *Phestilla sibogae* (Gastropoda, Opisthobranchia). *Comp. Biochem. Physiol. C* **101**, 299–305.
- Kempf, S. C., Page, L. R. & Pires, A. 1997 Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *J. Comp. Neurol.* **386**, 507–528. (DOI 10.1002/(SICI)1096-9861(19970929)386:3<507::AID-CNE12>3.0.CO;2-7.)
- Kuang, S. H. & Goldberg, J. I. 2001 Laser ablation reveals regulation of ciliary activity by serotonergic neurons in molluscan embryos. *J. Neurobiol.* **47**, 1–15. (DOI 10.1002/neu.1011.)
- Lacalli, T. C. 1981 Structure and development of the apical organ in trochophores of *Spirobranchus polycerus*, *Phyllodoce maculata* and *Phyllodoce mucosa*. *Proc. R. Soc. Lond. B* **212**, 381–402.
- Lacalli, T. C. 1984 Structure and organization of the nervous system in the trochophore larva of *Spirobranchus*. *Phil. Trans. R. Soc. Lond. B* **306**, 79–135.
- Lacalli, T. C. 1986 Prototroch structure and innervation in the trochophore larva of *Phyllodoce* (Polychaeta). *Can. J. Zool.* **64**, 176–184.
- Lacalli, T. C. & Gilmour, T. H. J. 2001 Locomotory and feeding effectors of the tornaria larva of *Balanoglossus biminensis*. *Acta Zool. (Stockholm)* **82**, 117–126. (DOI 10.1046/j.1463-6395.2001.00075.x.)
- Nielsen, C. 2001 *Animal evolution: interrelations of living phyla*, 2nd edn. Oxford University Press.
- Page, L. R. 2002 Comparative structure of the larval apical sensory organ in gastropods and hypotheses about function and developmental evolution. *Invertebr. Reprod. Dev.* **41**, 193–200.
- Page, L. R. & Parries, S. C. 2000 Comparative study of the apical ganglion in planktotrophic caenogastropod larvae: ultrastructure and immunoreactivity to serotonin. *J. Comp. Neurol.* **418**, 383–401. (DOI 10.1002/(SICI)1096-9861(20000320)418:4<383::AID-CN E2>3.0.CO;2-B.)
- Purschke, G. 1997 Ultrastructure of nuchal organs in polychaetes (Annelida)—new results and review. *Acta Zool. (Stockholm)* **78**, 123–143.
- Voronezhskaya, E. E., Hiripi, L., Elekes, K. & Croll, R. P. 1999 Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis*. I. Embryonic development of dopaminergic neurons and dopamine-dependent behaviors. *J. Comp. Neurol.* **404**, 297–309. (DOI 10.1002/(SICI)1096-9861(19990215)404:3<297::AID-CNE2>3.0.CO;2-I.)
- Voronezhskaya, E. E., Tyurin, S. A. & Nezlin, L. P. 2002 Neuronal development in larval chiton *Ischnochiton hakodadensis* (Mollusca: Polyplacophora). *J. Comp. Neurol.* **444**, 25–38. (DOI 10.1002/cne.10130.)
- Voronezhskaya, E. E., Tsitrin, E. B. & Nezlin, L. P. 2003 Neuronal development in larval polychaete *Phyllodoce maculata* (Phyllodoceidae). *J. Comp. Neurol.* **455**, 299–309. (DOI 10.1002/cne.10488.)

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