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Source: Zoological Science, 21(1) : 69-78

Published By: Zoological Society of Japan

Development and Neural Organization of the Tornaria Larva of the Hawaiian Hemichordate, *Ptychodera flava*

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ABSTRACT—We report scanning and transmission electron microscopic studies of the early development of the Hawaiian acorn worm, *Ptychodera flava*. In addition, we provide an immunohistochemical identification of the larval nervous system. Development occurs and is constrained within the stout chorion and fertilization envelope that forms upon the release of the cortical granules in the cytoplasm of the egg. The blastula consists of tall columnar blastomeres encircling a small blastocoel. Typical gastrulation occurs and a definitive tornaria is formed compressed within the fertilization envelope. The young tornaria hatches at 44 hr and begins to expand. The major circumoral ciliary band that crosses the dorsal surface and passes preorally and postorally is well developed. In addition, we find a nascent telotroch, as well as a midventral ciliary band that is already clearly developed. The epithelium of tornaria is a mosaic of monociliated and multiciliated cells. Immunohistochemistry with a novel neural marker, monoclonal antibody 1E11, first detects nerve cells at the gastrula stage. In tornaria, 1E11 staining nerve cells occur throughout the length of the ciliary bands, in the apical organ, in a circle around the mouth, in the esophageal epithelium and in circumpylorus regions. Axon(s) and apical processes extend from the nerve cell bodies and run in tracks along the ciliary bands. Axons extending from the preoral and postoral bands extend into the oral field and form a network. The tornaria nervous system with ciliary bands and an apical organ is rather similar to the echinoderm bipinnaria larvae.

Key words: hemichordate, tornaria, development, neural marker, larval nervous system

INTRODUCTION

The advances of molecular biology in the last two decades have added a new dimension to phylogeny. Hemichordates are again highlighted as key organisms to study the evolutionary diversification of body plans between the chordates and the invertebrates (Tagawa et al., 1998a; Tagawa et al., 2001). Hemichordates have been closely associated by their adult morphology with chordates as they exhibit gill slits and a dorsal hollow nerve cord formed by neurulation (Bateson, 1885; Morgan, 1891). The postulated homology of the stomochord of hemichordates with the notochord of chordates (Bateson, 1885) now seems unlikely (Balser and Ruppert, 1990; Peterson et al., 1999). The planktonic ciliated larval form of enteropneusts (acorn worms), the tornaria, resembles the echinoderm asteroid bipinnaria larva or the holothuroid auricularia larva establishing a strong phylogenetic connection between hemichordates and echinoderms (Bateson, 1884; Willmer, 1990; Nielsen, 1995).

One of the intriguing subjects in phylogenetic analysis has been the origin of the dorsal nervous system of chordates from ancestral deuterostome forms (Lacalli, 1996). Molecular studies in tornaria have shown the expression of orthologues of a number of vertebrate brain-specific regulatory genes occurs in larval neural structures (\(T\)-brain,
Tagawa et al., 2000; Otx, Harada et al., 2000; Distalless, Harada et al., 2001; Sox B, Taguchi et al., 2002; NK2.1, Takacs et al., 2002). The expression of some of these genes have also been studied in echinoderm larvae (Gan et al., 1995; Lowe and Wray, 1997; Shoguchi et al., 2000; Fuchikami et al., 2002). The failure of many genes to conserve their evolutionary function in the echinoderms with highly derived body organization (Lowe et al., 2002) warns that attention must be paid to the structure and cell diversity of each animal when making generalizations about the relationships between gene expression patterns and gene function.

In spite of a great deal of attention to the phylogenetic position of hemichordates, most of our knowledge of their development and structure was defined a number of years ago (Hyman, 1959) and there has been a paucity of recent work (Hadfield, 1975; Dautov and Nezlin, 1992; Benito and Pardos, 1997). To provide further structural information on the development of enteropneust tornaria larva, we undertook ultrastructural and immunohistochemical observations of early development and formation of the larval nervous system of *P. flava*.

**MATERIALS AND METHODS**

Adult acorn worms *P. flava* were collected from shallow sand bars at Kaneohe Bay or coral reefs at Paiko, Oahu Island, Hawaii. Mature eggs and sperms were obtained by induction of spawning using a shift of seawater temperature (Tagawa et al., 1998b). Fertilized eggs and embryos were reared at room temperature of 20 to 22°C.

For electron microscopy, eggs and embryos were fixed with 2.5% glutaraldehyde, 0.35M sucrose in 0.1M sodium cacodylate buffer, pH 7.4 for 1 hr at room temperature, postfixed with 1% OsO₄ in 0.1M cacodylate buffer and dehydrated through ethanol series. For the scanning electron microscopy (SEM), dehydrated samples were critical point dried, and then sputter coated with gold. Specimens were examined with a Hitachi S510 scanning electron microscope at 25kV. For transmission electron microscopy (TEM), ultrathin sections of epoxy resin embedded specimens were stained with uranyl acetate and lead citrate, and observed in a JEOL JEM 1001 electron microscope at 80kV. Four week old tornaria larvae were prepared by rapid freezing in liquid propane using a Leica KF80 apparatus and freeze substitution with 4% OsO₄ in acetone at –85°C.

For immunolocalization studies, embryos and larvae were fixed with 4% paraformaldehyde in sea water (SW) for 15 min at room temperature, washed with phosphate buffered saline (PBS, 0.1M, pH7.4) several times and briefly post-fixed with acetone at –20°C. Samples were stored in PBS containing 0.1% NaNO₃. The specimens were incubated in culture supernatant of 1E11 hybridoma cells and/or rabbit anti-serotonin polyclonal antibody (diluted 1/1,000 in PBS, DiaSorin, USA) overnight in a refrigerator or 2 hr at room temperature. After several rinsing with PBST (PBS containing 0.1% Tween 20), samples were treated with secondary antibodies labeled with Alexa 488 and/or Texas Red (Molecular Probes, Oregon, USA). Samples were observed with a confocal laser scanning microscope (Olympus Fluoview V300).

The monoclonal antibody, 1E11, was developed in mice immunized with an extract of radial nerve of the starfish, *Asterina pectinifera*. Fused hybridoma cell supernatants were screened for staining of the known nervous system of the bipinnaria larva of the same species. The 1E11 monoclonal antibody recognizes unreported adult and larval echinoderm nerve tracks as well as those that have been previously described (Nakajima et al., in press).

**RESULTS**

**Ultrastructure of early development**

SEM examination shows the egg is invested in a fibrous vitelline envelope (Fig. 1a, b). When this is removed the outer surface of *P. flava* unfertilized eggs is covered with dense, spiky microvilli, 1 to 2 µm in length (Fig. 1b). TEM micrographs of sections of unfertilized eggs show a 1 to 2 µm deep cytoplasmic cortical layer at the surface of the egg with 0.3 µm electron-dense cortical granules arrayed just beneath the plasma membrane (Fig. 1c). Yolk granules (YGs) occupy most of the endoplasm of the egg below the cortical layer. The YGs have an electron dense region and a relatively electron-lucent region of fine meshwork. Other than YGs, the cytoplasmic organelles are poorly developed and only scattered mitochondria, electron-dense lysosomal granules, and free ribosomes are observed.

Upon fertilization, the fertilization envelope forms, meiosis occurs and the egg cleaves into 2 equal blastomeres at 3 hr (Fig. 1e). In the TEM, the fertilization envelope consists of a thin electron-dense outer surface layer and a thicker 0.2 to 0.3 µm underlying amorphous layer (Fig. 1d). A similar, but more electron-lucent substance fills the perivitelline space. The cortical layer at the surface of the unfertilized eggs is no longer present in fertilized eggs. Dense microvilli cover the surface of the blastomeres just as they covered the surface of the unfertilized egg (Fig. 1e).

After first cleavage, cell divisions continue about every 30 min to produce blastulae within 12 hr. The first 3 cleavages occur equally, the fourth cleavages appear unequal as previously described by Tagawa et al. (1998b). However, by blastula stage, the blastomeres look relatively similar in size as seen in an SEM image of a blastula still enclosed in the fertilization envelope (Fig. 2a). Eggs and embryos are opaque with yolk which makes discerning the internal details difficult with light microscope observations. To investigate the internal structure, fixed embryos were split with a tungsten needle and observed by SEM as shown in Fig. 2. The blastomeres of blastula are tall columnar cells 35 to 45 µm in height and 5 to 15 µm in width. The blastocoel is very small; it is only about 20 µm in diameter (Fig. 2b).

SEM observations of split beginning gastrulae show the ectodermal cells flattened to about 20 µm in height while the vegetal plate cells are elongated into the blastocoel to a height of about 50 µm (Fig. 2c) almost filling the blastocoel with the archenteron cells (Fig. 2d). Gastrulation appears to be a continuation of this process, apparently adapted to morphogenesis in the tight chorion, with the elongating archenteron cells filling the blastocoel and ultimately forming a tight cleft, which is the gut opening. TEM images of the gastrula stage ectodermal cells show that the yolk granules align in the apical region of the cells and nuclei localize just...
The cytoplasm is filled with a range of electron-lucent to moderately electron-dense granules. The embryos remain constricted in the tight chorion and fertilization membrane until they hatch as an early tornaria larva at 44 to 45 hr. Just after hatching, the larva is dense and ovoid (Fig. 2e, f) with the major ciliary bands and the nascent telotroch of the definitive tornaria already visible on the surface. By 5 days, three days after hatching, the larva has increased considerably in size due to expansion of blastocoel space. During this expansion the epithelial cells flatten, and the tornaria becomes more angular in shape (compare Fig. 2e with Fig. 3a) and optically transparent (Tagawa et al., 1998b). The major ciliary surface features of the tornaria can best be discerned and will be described in context of SEM images of the 5 day swimming tornaria (Fig. 3), but these features are all evident in the just-hatched tornaria (Fig. 2e, f).

**SEM observation of tornaria larvae**

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**a) circumoral ciliary band**

The major ciliary band of the larva loops twice around the larval body (Fig. 3a, c) crossing at the apical organ on the anterior dorsal surface (Fig. 3d) and connecting ventrally via preoral and postoral sections that pass, respectively, anterior and posterior of the mouth.

**b) dorsal anterior apical organ and apical tuft**

The apical organ forms a flattened plate on the anterior apex of the tornaria (Fig. 3d.). The circumoral ciliary bands looping around the larval body join at it four corners. The apical tuft of long cilia emerges from its center with the eye-spots evident as grooves to the left and right.
c) perioral ciliary band

The mouth opening is filled with cilia from the perioral ciliary band that tightly encircles the opening of the mouth (Fig. 3c). As will be shown below, the ring of nerve tracks associated with the perioral band is a significant component of the larval nervous system.

d) midventral ciliary band

A midventral ciliary band extends from a dense zone of cilia surrounding the anus and connects anteriorly with the transverse postoral ciliary band (Fig. 3e). Among the zone of cilia around the anus, there are two distinct round depressions that have not previously been noticed and are to the right and left and slightly dorsal of the anus (Fig. 3e). As the midventral ciliary band reaches the postoral ciliary band, it branches to the right and the left. All three branches join the post oral band and delimit two round areas of non-ciliated cells (Fig. 3e).

e) telotroch

A nascent telotroch is present on the earliest larvae, forming a dorsally projecting circle around the anus at the posterior end of the larva (Fig. 3b).

In other features of note, the dorsal ectoderm is a mosaic of cells, some with a single cilium and some with multiple cilia (Fig. 3c). The cells on the inner surface of stomach and intestine are also multiciliated (data not shown).

Looking into the inside of a tornaria that has been broken open, there is a matted basal lamina on the blastocoelar surface of the ectodermal cells (Fig. 3f). The blastocoelar space contains a fine fibrous matrix with a few mesenchyme cells associated here and there.

The nervous system of tornaria larvae

The nervous system of P. flava has not been studied well immunohistochemically. The staining with anti-serotonin antibodies of the neurons in the apical organ of the tornaria larva in this Hawaiian species is the only reported study (Tagawa et al., 2001). We examined the histochemistry with a novel neural marker, monoclonal antibody 1E11, developed with echinoderm neural tissue as antigen (Nakajima et al., in press). As shown on the Fig. 4a, cells immunoreactive to 1E11 are present in the apical organ and along the ciliary bands. Prominent immunoreactive nerve tracks are evident.
Development and Nervous System of Tornaria

The staining of the nerve track around the perioral ciliary band is prominent (Fig. 4a, double arrows). In the major circumoral ciliary bands, the axons that extend along the base of the ciliary bands, the nerve bodies and the apical processes of the nerve cells that extended along the outer surface of the ciliary bands (Nakajima, 1986b) are all immunoreactive. Consequently, this ciliary band appears as ladder-like structures of two lines connected periodically with nerve cell bodies (Fig. 4a). Ultrastructurally, the ciliary band consists of epidermal cells that are characterized by a conspicuous nucleus with dense heterochromatin, electron-dense granules and vacuoles. In the basal part of the ciliary band, there is a bundle of nerve track consisting of 20 to 30 axons (Fig. 5b).

Fine immunoreactive nerve tracks project into the oral field from cells in the ciliary band (Fig. 4a, e) creating a neural network in the oral field. Some cells in the stomodeal and esophageal epithelial cells and cells around the pyloric sphincter also exhibit 1E11 reactivity (Fig. 4f). Immunofluorescence is also seen in the nascent telotroch (Fig. 4a).

Cells in the apical organ are highly reactive to 1E11. We examined double staining with anti-serotonin and 1E11 antibodies of neural cells in the apical organ. Staining is first detected in the apical epidermis of the late gastrula around 40 hr of development. At this stage one or two cells stain and often they are both serotonergic and 1E11-reactive (Fig. 4g, h, i). In later tornaria, serotonergic cells and cell processes appear in the apical plate and in a restricted region of the adjacent anterior ciliary bands (Fig. 4c). These cells plus other are immunoreactive with 1E11 (Fig. 4b, c). Numerous nerve cells in the apical organ react to 1E11 (Fig. 4b), a sub-
set of these also expresses serotonin (Fig. 4c, d).

We examined the ultrastructure of the apical organ by TEM. It consists of a cluster of tall nerve cells, supporting epithelial cells and a large mass of numerous axon tracks (Fig. 5a, c). Epidermal cells and nerve cells are connected to each other by septate junctions at their apical ends (Fig. 5, inset). The nuclei of nerve cells are less dense and larger than those of epidermal cells. The axons in the apical organ accumulate a large number of 30 to 50 nm diameter synaptic vesicle-like inclusions with a variety of profiles that may be characterized as dense cored, cored, or open. Patent synaptic endings are not observed.
DISCUSSION

Early development of *P. flava*

The SEM and TEM observations of early development of *P. flava* reported in this paper confirm, increase and revise our knowledge from previous studies of tornaria (Colwin and Colwin 1953; Hyman, 1957; Hadfield; 1975; Tagawa et al., 1998b) that were mostly based on the light microscopic observations.

The details of fertilization processes of *Saccoglossus kowalevskii*, a direct developing acorn worm were reported by Colwin and Colwin (1963a, b). As in *S. kowalevskii*, the fertilization envelope of *P. flava* is derived from an inner layer of the unfertilized egg envelope with the addition of a fertilization envelop apparently derived from the release of the cortical granule contents upon egg activation. Although a cortical layer has not been previously observed in the unfertilized egg of *P. flava* (Hadfield, 1975; Tagawa et al., 1998b), the TEM images show clearly the existence of a distinct cortical layer with unique electron dense cortical granules. This specialized layer with its granules disappears upon fertilization. The outer and inner envelopes of the unfertilized egg remain as chorion and fertilization envelope respectively during cleavage, blastula, gastrula and early larval stages (Tagawa et al., 1998b). Unlike echinoderm embryos, which hatch and start swimming as early embryos, the *P. flava* embryo remains enclosed in the very tight chorion until it is a fully developed tornaria. The diameter of blastula stage is at most 10% larger than that of unfertilized egg allowing for only a very limited blastocoel. The topology of gastrulation with the differentiation of thinner ectodermal cells and elongated vegetal plate cells to achieve gastrulation also appears to be an adaptation to morphogenesis to this restricted space. Even when the tornaria hatches it is still compressed and dense and only expands after escape from the tight chorion. It has been reported, based on light microscopic observation, that *P. flava* hatches at late gastrula stage (Tagawa et al., 1998b). Our results with SEM show that the larva just after hatching is a definitive tornaria with its specific ciliary band arrangement.

The multiciliated cell cluster and cell alignment at the posterior region of the young *P. flava* tornaria appear to precursors of telotroch and midventral ciliary band, respectively, as they appear to be in *Balanoglossus biminiensis* (Lacalli and Gilmour, 2001). These structures have not been recorded in *P. flava* by prior light microscopic observations. Although Lacalli and Gilmour (2001) report that a well developed nerve cord exists under the telotroch of *B. biminiensis* tornaria, we did not detect any significant immunohistochemical staining with 1E11 that might appear to be a nerve track under the telotroch of the *P. flava* tornaria for at least the first 7 days of development.

Nervous system

One of the interesting questions concerning tornaria morphology is the transition of the larval nervous system to the adult nervous system, especially because the transition from tornaria larva to juvenile acorn worm does not involve the loss of larval structures or cells (Agassiz, 1873). There are several previous reports concerning the ultrastructure of the nervous system and related structures of tornaria (Bran-denburger et al., 1973; Nielsen, 1987; Dautov and Nezlin, 1992; Lacalli and West, 1993; Lacalli and Gilmour, 2001). Anti-serotonin antibody, which has been the most broadly applied for showing larval nervous system structures, reacts to the apical organ with nerve tracks extending into oral hood (Fig. 4c, Tagawa et al., 2001). Ultrastructural observations that show nerve cell bodies along the whole length of ciliary bands and axons running along the basal part of ciliary bands do not stain with anti-serotonin (Dautov and Nezlin, 1992; Lacalli and West, 1993; Lacalli and Gilmour, 2001). Using the novel neural marker 1E11, we can visualize a much more complete complement of the nervous system of young tornaria. 1E11 reveals the neural structures already reported by anti-serotonin and electron microscopy as well as previously undescribed neural structures.

The details we have been able to add to the description of the nervous system of tornaria show that it even more resembles that of asteroid bipinnaria larva (Nakajima et al., in press). As has been noted previously (Lacalli and West, 1993; Lacalli, 1996), a number of features are very similar. The flask shaped nerve cells evenly spaced along the ciliary bands, with axons extending from the base of the nerve cells and apical processes extending from the apex of these cells along the ciliary bands appear identical. Fine nerve filaments derived form nerve cells in the ciliary bands extend into oral field and form a network in both sets of larvae. They both exhibit a nerve plexus in esophageal epithelium.

There are some contrasting features of the apical organ/apical ganglion between the two groups. The tornaria has an apical organ with eyespots at the apical tip of the body and contains both serotoninergic and 1E11 positive nerve cells. In the bipinnaria the ganglia containing serotoninergic, anti-GFNSALMFamide (S1)- and 1E11-positive cells are bilateral and are in the more proximal ciliary band of the oral hood (Nakajima, 1988; Moss et al., 1994, Nakajima et al., in press). In the echinoid pluteus, the apical ganglion containing serotoninergic, S1- and 1E11-positive cells appears at the apical tip between the anterolateral arms (Nakajima, 1986a; Bisgrove and Burke, 1987; Yaguchi et al., 2000; Beer et al., 2001; Nakajima et al., in press). The similarities and shifts in the location of apical neural structures between hemichordate, echinoid and asteroid larvae are intriguing. The future study of the apical ganglion in the holothuroid auricularia larva, which is more similar to the tornaria in morphology, will provide important information.

Our results here provide the evidence that hemichordate and echinoderm larval nervous systems resemble each other and support current molecular phylogeny that these two phyla are sister groups. In spite of these larval similarities, the later development of these two phyla is quite distinct. The bilateral body plan of the enteropneust hemi-
chordate larvae is maintained as the larva transitions into the adult, while echinoderm larvae totally reorganize from their bilateral larval form into the radial adult form. The comparative study of the nervous system during metamorphosis of these animals remains as a very interesting issue and its further study will contribute to our understanding of the evolution of the nervous system in deuterostomes. The new neural marker 1E11 promises to contribute significantly to such studies. Assuming larval nervous systems of hemichordates and echinoderms share the common ancestral feature(s) with the central nervous system of chordates, it will be especially interesting to use this antibody to study the developmental stages of chordates, the third phylum of deuterostomes.

ACKNOWLEDGMENTS

YN was supported by Keio Gijuku Academic Development Funds and KT was supported by American Cancer Society Institutional Grant 436328. TH was supported in part by a Special Visiting Professor MEXT Award of the Japanese government through Tokyo Medical and Dental University. We express our thanks to Dr. T. C. Lacalli for important advice and comments on the manuscript and to Dr. R. D. Burke for critical reading and comments of the manuscript.

REFERENCES

Bateson W (1885) The later stages in the development of B. kowalevskii, with a suggestion as to the affinities of the Enteropneusta. Quart J Micr Sci 25(Supp): 81–122
Lacalli TC, West JE (1993) A distinctive nerve cell type common to diverse deuterostome larvae: Comparative data from echinoderms, hemichordates and amphioxus. Acta Zool (Stockh) 74: 1–8


(Received September 9, 2003 / Accepted September 24, 2003)