Evolution of Developmental Control Mechanisms

Neural development in *Eucidaris tribuloides* and the evolutionary history of the echinoid larval nervous system

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ARTICLE INFO

ABSTRACT

The structure and development of the larval nervous systems of all classes of echinoderms have been described and details of embryonic signaling mechanisms patterning neurogenesis have been revealed experimentally in sea urchins. Several features of neuroanatomy and neural development indicate that echinoids are the most derived group. Here we describe the development and organization of the nervous system of a cidaroid, *Eucidaris tribuloides*. The cidaroids are one of two major clades of echinoids, and are considered to have features of anatomy and development that represent the common ancestor to all echinoids. The embryos of *E. tribuloides* lack a thickened animal plate and serotonergic neurons arise laterally, associated with the ciliary band. Although lacking a discrete apical organ, plutei have serotonergic neurons associated with the pre-oral ciliary band joined by a few diffusely arranged connecting axons. Chordin and Hnf6, early markers for oral ectoderm and ciliary band, are expressed in similar patterns to euechinoids. However, an animal pole domain marker, Nk2.1, is expressed in a broader region of anterior ectoderm than in euechinoids. Six3, a proneural marker that is restricted to the animal plate of euechinoids, is expressed laterally in the preoral ciliary band at the same location as the serotonergic neurons. We conclude that the organization and development of the larval nervous system of *E. tribuloides* retains features shared with other echinoderm larvae, but not with euechinoids. These data support a model in which several distinctive features of euechinoid neural organization are derived, having arisen after the divergence of the two clades of echinoids about 265 million years ago. We hypothesize that differences in the developmental mechanisms that restrict neurogenesis to the animal pole forms the basis for the distinctive neuroanatomy of euechinoids.

Introduction

Detailed descriptions of the neuroanatomy and neural development of larval echinoderms provide an opportunity to understand the evolutionary history of these distinctive deuterostomes (Angerer et al., 2011). Although each class has a characteristic feeding larval form, their nervous systems are, overall, very similar (Burke, 1983; Chee and Byrne, 1999; Nakajima et al., 2004a; Nakano et al., 2006; Bishop and Burke, 2007; Hirokawa et al., 2008; Dupont et al., 2009). In all larval echinoderms that feed, neurons and tracts of interconnecting axons are associated with the ciliary bands and musculature of the esophagus. As well, all of the larval forms have serotonergic neurons at the anterior end that have been described as apical organs (reviewed in Byrne et al., 2007). Despite these overall similarities, the larvae of sea urchins have a number of distinguishing features. The apical organ of euechinoids originates

from the animal plate of the gastrula and forms a thickened disc in which there are two bilateral clusters of neurons and a robust connecting neuropil. The apical organ is separate from the ciliary band and constitutes a discrete anterior sensory ganglion. As well, euechinoids are distinctive in having paired lateral ganglia that project an array of axons toward the posterior end of the larva. Given the phylogenetic relationships of the echinoderm classes (Smith et al., 2004), these features of neural organization suggest the sea urchin larva is the most derived of the echinoderm larval forms (Byrne et al., 2007; Dupont et al., 2009).

Echinoids are a diverse group with a range of adult and larval forms (Wray, 1992; Kroh and Smith, 2010). On the basis of morphology or molecular phylogenies, urchins divide into two major groups: the cidaroids and the euechinoids (Smith et al., 2004, 2006). The euechinoids are the largest group, containing the more familiar forms of sea urchins and sand dollars. The second group, the cidaroids, or pencil urchins, are indistinguishable from fossil urchins that are more than 250 million years old. Cidaroids have a number of features of their development that set them apart from euechinoids. Cleavage stage embryos have an irregular

number of micromeres and primary mesenchyme forms after archenteron invagination (Schroeder, 1981; Wray and Mcclay, 1988). In addition, some cidaroid species lack the thickened animal plate and tuft of immotile cilia at the anterior end that is typical of euechinoids (Schroeder, 1981; Bennett et al., 2012). There are excellent images and drawings of cidaroid larvae in which a discrete apical sensory organ is not apparent (cf. Mortensen, 1937; Schroeder, 1981; Emlet, 1988). To determine if the apical organ is derived from within the euechinoids, we have examined the development and organization of the larval nervous system of *Eucidaris tribuloides*. Remarkably, its larval nervous system has a number of features that make it more similar to the nervous systems of a larval sea cucumber or sea star. These observations enable us to date the origin of several features of echinoid neural organization to the divergence of cidaroids and euechinoids.

Materials and methods

Gametes and culture of embryos and larvae

Adult *E. tribuloides* were collected by snorkeling on reefs flats on the island of Bocas del Toro, Panama, or were purchased from Gulf Specimens Marine Lab and held at room temperature in natural seawater. Gametes were obtained by intracoelomic injection of 0.5 M KCl and embryos were raised in 0.2 μm filtered seawater (FSW) at 25 °C. Larvae were cultured in 4 L glass jars according to Bishop and Brandhorst (2007). In some instances embryos were treated with LiCl (30 or 60 mM), $ZnSO_4$ (125, 250, or 500 μM) or 1-azakenpaullone (1 μM, Sigma). Embryos were transferred to untreated control, 1% DMSO, or experimental solutions after first cleavage and cultured. Embryos were returned to normal sea water after 42 h or 68 h and cultured until they were fixed at 56, 68, 72, 96, or 120 h. *Strongylocentrotus purpuratus* were collected near Victoria, BC, and *Lytechinus variegatus* were purchased from Gulf Specimens Marine Lab.

Immunofluoresence preparations

Embryos and larvae were harvested by centrifugation and fixed for 10–15 min in 100% methanol, or 4% formaldehyde formulated in FSW, or PEM (4% paraformaldehyde in 100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% Triton X-100, pH 6.8) (Vielkind and Swierenga, 1989), washed with PBS-Tween (0.02% Tween 20), resuspended in blocking buffer for 30 min (Superblock, Thermo Scientific) containing 0.03% Triton X-100, and incubated at 4 °C overnight in primary
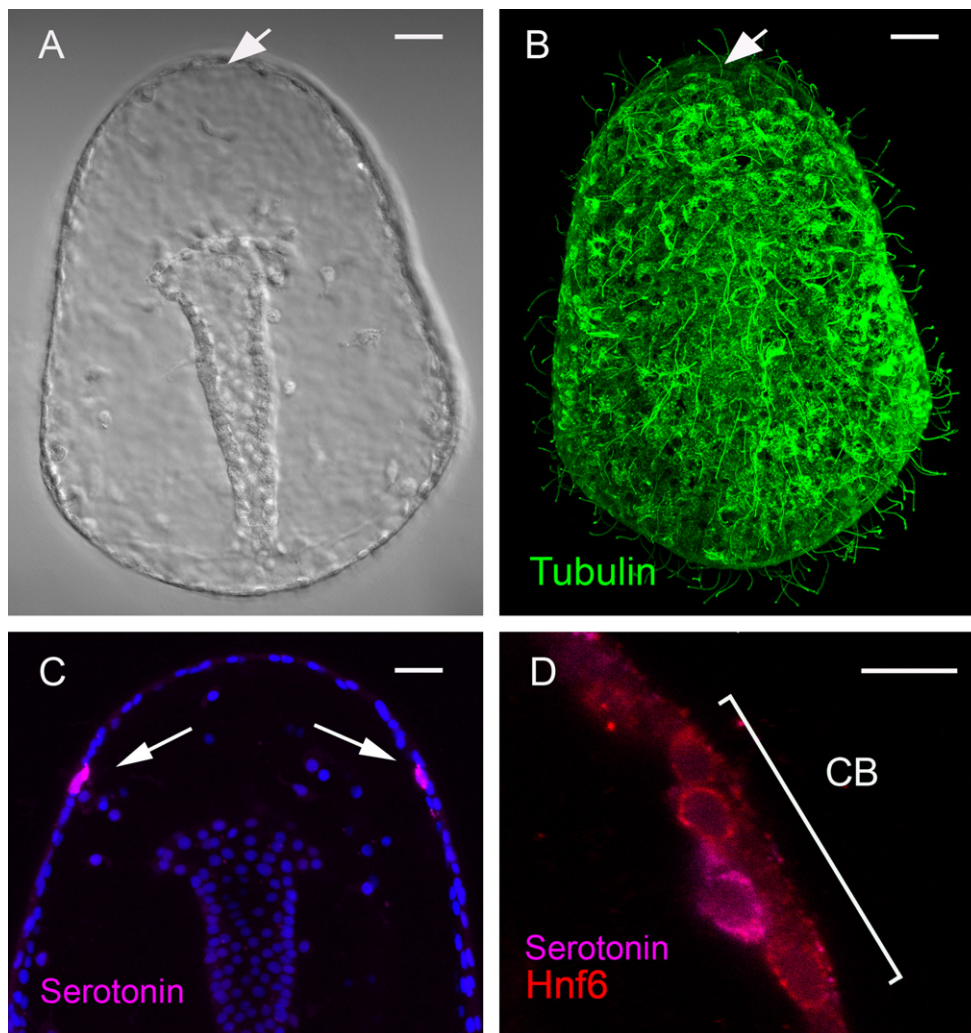
Fig. 1. (A,B) *E. tribuloides* embryos lack a thickened animal plate and elongate, non-motile cilia. DIC optical section (A) and maximum intensity projection of a stack of confocal images (B) of a 48 h embryo prepared with anti-tubulin indicate that the epithelium at the animal pole of the embryo is of uniform thickness and does not have a ciliary tuft (arrows). (C) Single optical section of 48 h embryos 2 or 4 cells that express serotonin are located in bilaterally symmetric positions in the region of the developing ciliary band. (D) A 48 h embryo prepared with antibodies against serotonin and Sp-Hnf6 indicates the serotonergic neurons arise associated with the ciliary bands. Bars=10 μm.

antibody diluted in blocking buffer. Embryos were rinsed three times in PBS-Tween (15 min), incubated in secondary antibody diluted in blocking buffer for 60 min at room temperature, and rinsed three times in PBS-Tween. Specimens were mounted 1:1 in Slowfade® Gold Antifade Reagent with DAPI (Invitrogen) and images were collected using Zeiss LSM 700 confocal microscope. Antibodies used were anti-synaptotagmin (1E11, Nakajima et al., 2004a), anti-serotonin (Sigma), anti-Hnf6 (Yaguchi et al., 2010a), anti-Sp-chordin (Burke, unpublished), anti-SpNk2.1 (Takacs et al., 2004), anti-SpSix3 (Burke, unpublished), anti-tubulin (Santa Cruz Biotechnology), Goat anti-Rabbit, Alexa Fluor 488 and 568 (Invitrogen), Goat anti-Mouse Alexa Fluor 488 and 568 (Invitrogen), Goat anti-Rat 488 and 635 (Invitrogen). Image size was adjusted, contrast and brightness were corrected, and images were assembled into figures using Photoshop CS2 (ver. 9.0.2).

Results

Neural ontogeny

The blastoderm and ectoderm of *E. tribuloides* blastulae and gastrulae are uniform in thickness at the animal pole and embryos lack the characteristic tuft of non-motile cilia (Fig. 1A). Forty-eight hour gastrulae, prepared with antibodies to tubulin, are covered by cilia of uniform length (Fig. 1B). In embryos between 48 and 56 h,

the thickened ridges of ciliary band begin to form, and cells that are immunoreactive to anti-serotonin appear in symmetric positions on the lateral aspect of anterior ectoderm in association with the developing ciliary band (Fig. 1C and D). As in other species of sea urchin, ciliary band cells in *E. tribuloides* express the transcription factor Hnf6 (Otim et al., 2004; Poustka et al., 2004) and can be identified at this stage by a thin rim of nuclear immunoreactivity (Fig. 1D). The neurons containing serotonin underlie the ciliary band cells and project a ciliated apical end through the overlying epithelium. Thus, *E. tribuloides* embryos lack a thickened animal plate and serotonergic neurons arise in association with the anterolateral ciliary band.

In prisms (72 h), neurons that are immunoreactive with anti-synaptotagmin appear adjacent to the ciliary band (Fig. 2A). Initially there are five cells in the ventral transverse band that express synaptotagmin. These cells project axons with a terminal swelling or growth cone. The midline cell projects axons laterally within the ventral transverse band and the two most lateral cells project axons toward the anterior end and medially, also within the ciliary band. Two cells that are at the base of the post-oral arms project axons medially within the ciliary band (Fig. 2A). As well, these two post-oral neurons have distinctive axonal projections that are directed anteriorly under the oral ectoderm and re-enter the ciliary band lateral to the mouth. Neurons expressing synaptotagmin also appear in the ciliary band lateral to the mouth. These neurons project axons anteriorly and posteriorly within the ciliary band. In early plutei (92 h–141 h), the
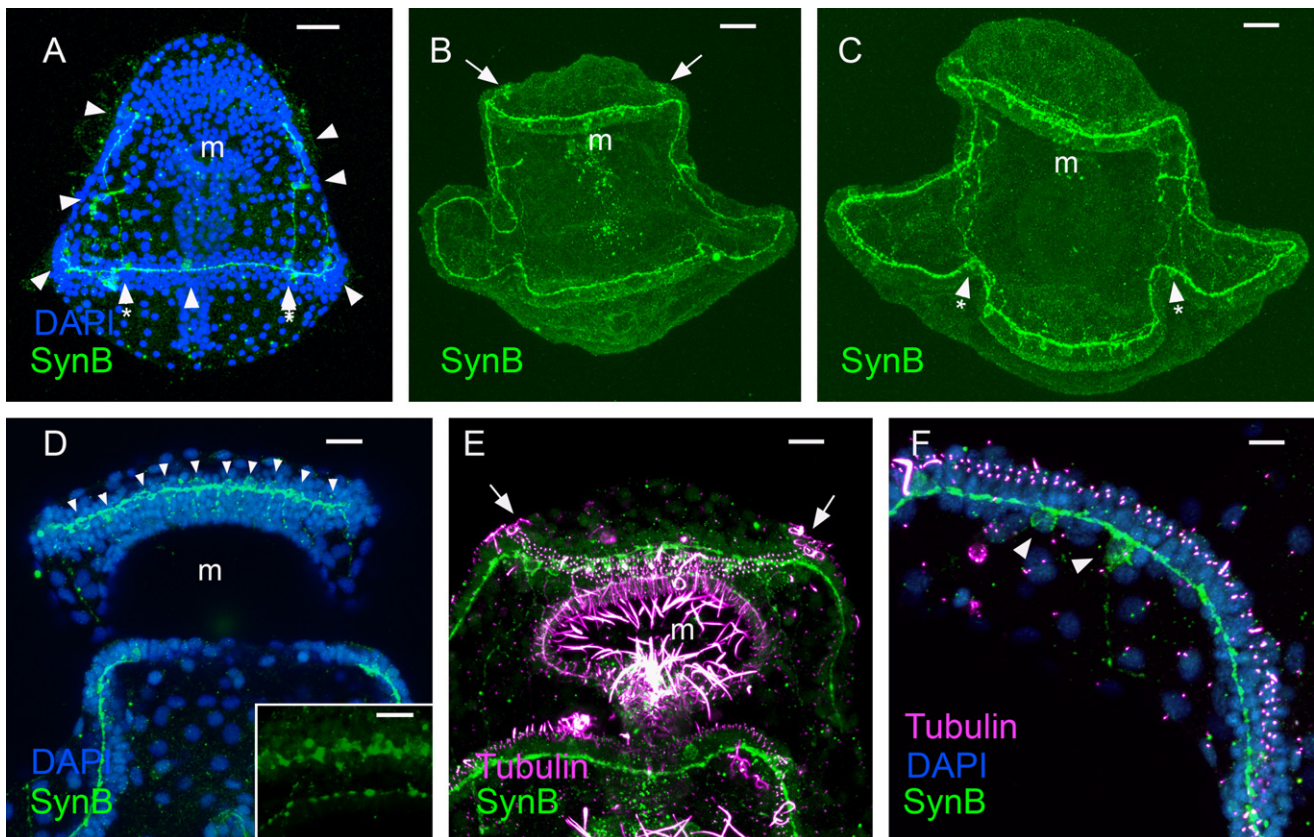

Fig. 2. Neurogenesis in prism and early pluteus larvae of *E. tribuloides*. (A) In 72 h prism larvae, the ciliary bands have synaptotagmin containing neurons projecting neurites with terminal growth cones. Arrowheads indicate neural cell bodies. There are five synaptotagmin neurons in the ventral transverse band and two neural cell bodies in each of the lateral ciliary bands. The cells that will be located at the base of the post-oral arms (post-oral neurons, *) project axons within the ciliary band and form a distinctive axonal projection that is directed anteriorly under the oral ectoderm. (m, mouth). (B–F) Confocal images of larvae (92–141 h) prepared with anti-synaptotagmin and anti-tubulin. (B) In the early pluteus, the nervous system within the ciliary band encircles the oral field. Bilaterally on the oral hood there is a small cluster of synaptotagmin containing neurons (B,E arrows) that lie on the aboral side of the ciliary band. These cells have curled apical cilia and are in the location of the serotonergic cells. (C) There are axonal projections beneath the oral field, most clearly associated with the post-oral neurons (*). (D) In the pre-oral transverse band there are a series of closely packed neurons that interconnect and project axons toward the adjacent adoral ciliary band. Inset: a 3D projection of pyramidal cells in the preoral ciliary band. Neurons in other regions of the ciliary band (F) are more widely spaced, and lie on the oral side of the ciliated cell, and project axons with the basal tracts or beneath the oral ectoderm. Bars=10 μm.

nervous system within the ciliary band completely encircles the oral field (Fig. 2B). There are axonal projections beneath the oral field, most clearly associated with the post-oral neurons (Fig. 2C). Bilaterally on the oral hood there is a small cluster of neurons that lie on the aboral side of the ciliary band. This cluster of neurons has cilia that are curled and lie on the surface of the epithelium (Fig. 2B and E). In the pre-oral transverse band, there are a series of closely packed neurons that interconnect and project axons toward the adjacent adoral ciliary band (Fig. 2D). The neurons are pyramidal in shape, with a small apical projection to the surface and expanded bases that contact adjacent neurons (Fig. 2D and inset). In contrast, neurons in other regions of the ciliary band (Fig. 2F) are more widely spaced, lie on the oral side of the ciliated cells, and project axons with the basal tracts or beneath the oral ectoderm. Thus, morphologically distinct neurons arise throughout the ciliary band and interconnect by projecting axons in tracts within the ciliary band.

Neural markers

Several molecular markers for specific regions of embryonic ectoderm have been identified in urchins and comparisons of their distribution can reveal similarities in ectoderm patterning. Hnf6 is a transcription factor expressed in the ciliary band of other echinoids (Poustka et al., 2004; Otim et al., 2004). In *E. tribuloides*, an antibody against Hnf6 indicates that the protein is initially expressed broadly throughout the ectoderm and between 48 and 64 h it begins to concentrate in scattered oral ectodermal cells and in the ciliary band (Fig. 3A). The ciliary band cells have Hnf6 in the cytoplasm and at the periphery of nuclei, whereas it is nuclear in other echinoid species (Yaguchi et al., 2010a). In euechinoids, Nk2.1 is initially expressed only in the animal pole domain and subsequently spreads to include an anterior domain of oral ectoderm (Takacs et al., 2004). In *E. tribuloides* late gastrulae (52 h), Nk2.1 is expressed in a broad region of apical ectoderm including an anterior region of oral ectoderm, the pre-oral ciliary band, and aboral ectoderm at the animal pole (Fig. 3B). In early plutei (72 h), Nk2.1 is expressed in the dorsal esophagus, a feature similar to the expression in other species (Fig. 3C). Chordin (Chd) is part of the oral gene regulatory network and expressed in a posterior subdomain of the oral ectoderm (Bradham et al., 2009; Saudemont et al., 2010). In 52 h embryos, Chd localizes to a single brightly fluorescent region adjacent to the nucleus of cells in the central oral ectoderm. As in euechinoid species, it is restricted to a region between the mouth and the post-oral transverse ciliary band. In euechinoids, the proneural transcription factor Six3 is expressed in the animal pole domain (Poustka et al., 2007; Wei et al., 2009). *In situ* hybridizations
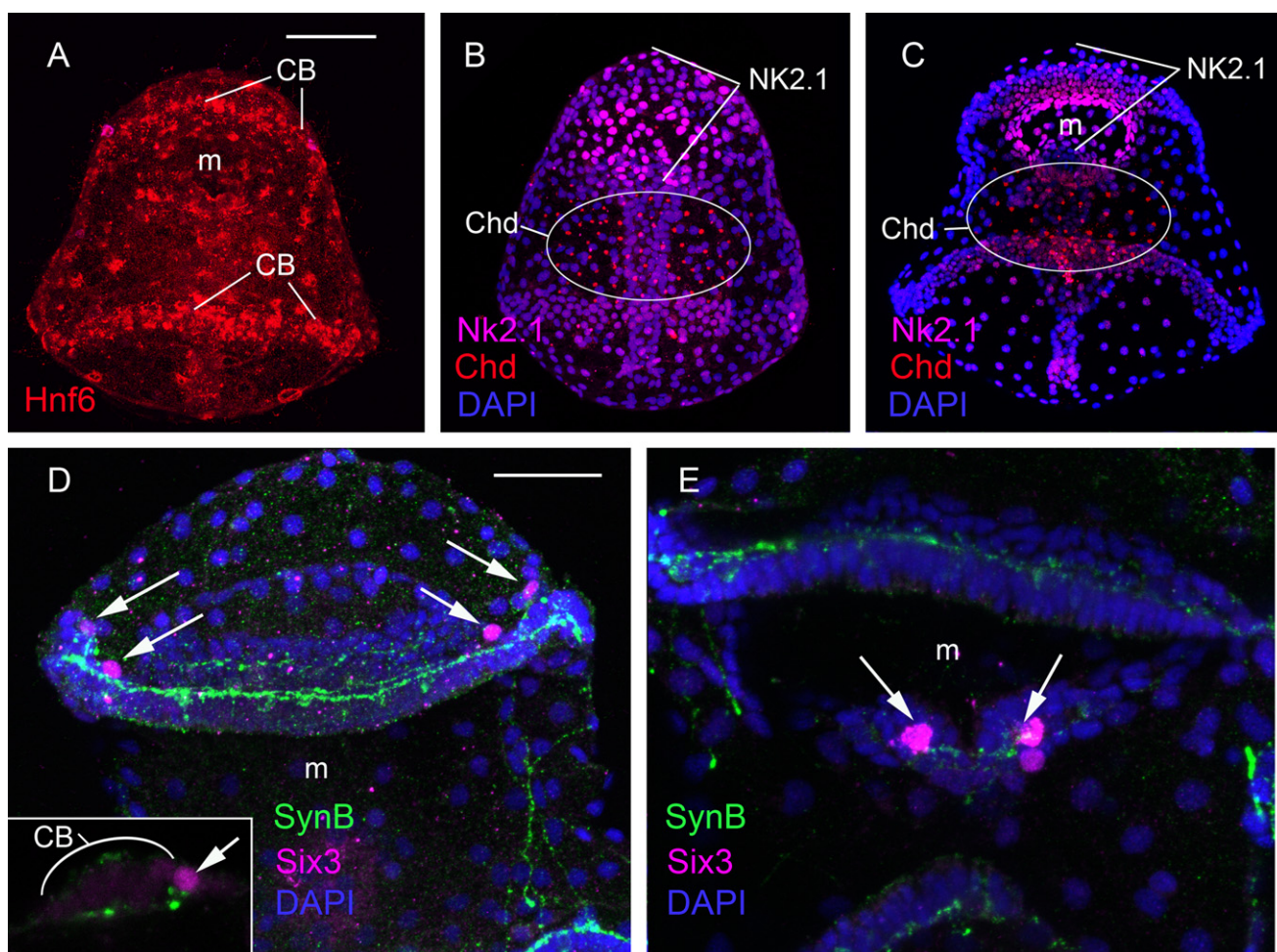

Fig. 3. Expression of ectodermal antigens of ciliary band, oral ectoderm, and anterior neural ectoderm in *E. tribuloides* embryos. A. 64 h embryo prepared with anti-Hnf6 in the ciliary band. (B) 52 h, late gastrula prepared with anti-Nk2.1, which is expressed in a broad region of anterior ectoderm. Chordin (Chd) localizes to a single brightly fluorescent region adjacent to the nucleus of cells in part of the oral ectoderm. (C) 72 h early pluteus larva prepared with anti-Nk2.1 and anti-Chd. Nk2.1 is expressed in anterior neural ectoderm and the dorsal esophagus; a feature similar to the expression in other species. Chd continues to be expressed in a subdomain of the oral ectoderm. (D,E) 92 h larvae prepared with anti-synaptotagmin and anti-Six3. Six3 immunolocalizes to nuclei of a small number of cells in two lateral locations of the pre-oral transverse ciliary band and the lower lip of the mouth. Inset: optical cross-section detail of Six3 expressing neuron associated with the ciliary band on the oral hood of 92 h embryo. Bars=20 μm.

indicate that expression is initially in the thickened animal plate of blastulae and then becomes restricted to a ring of cells in the animal plate of gastrulae (Wei et al., 2009). In later stages, Six3 is expressed in cells adjacent to the mouth and the margins of the apical organ (Burke, unpublished). In *E. tribuloides*, Six3 protein immunolocalizes to nuclei of a small number of cells in the lateral region of the pre-oral transverse ciliary band and the lower lip of the mouth (Fig. 3D and E). These markers indicate that in *E. tribuloides* the molecular basis of patterning of ectoderm into ciliary band and oral ectoderm is similar to that in euechinoid urchins. However, Nk2.1 has a broader apical distribution and Six3 is not restricted to a small apical domain but is expressed in the two lateral regions where the serotonergic neurons arise.

Larval nervous system

There are several features of the neuroanatomy of the larval nervous system that distinguish euechinoid urchins from other echinoderms, particularly the discrete apical organ and lateral ganglia neurons. Initially, plutei of *E. tribuloides* have paired anterolateral and post-oral arms, typical of four-arm echinoplutei. After two weeks of development, the pre-oral arms appear and subsequently the posterodorsal pair of arms are added. Serotonergic neurons occur in two small clusters on the lateral aspect of the pre-oral hood between the bases of the anterolateral and pre-oral arms (Fig. 4A, E and D). There are three or four flask-shaped serotonergic neurons in each cluster, and no additional synaptotagmin immunoreactive neurons. The serotonergic neurons are closely associated with the ciliary band. In fully developed eight-arm plutei, the clusters of serotonergic neurons remain relatively small, four or five neurons in each (Fig. 5A–C). An indistinct anti-serotonin immunoreactive commissure consisting of a few individual axons extends between the two clusters (Fig. 5C). Axons project posteriorly from these clusters, but they are not within the ciliary band axonal tracts (Fig. 5A). Serotonergic axons extend lateral to the gut, apparently within the blastocoelar space. Additionally, eight to ten individual serotonergic neurons are found within the ciliary bands and these project axons within
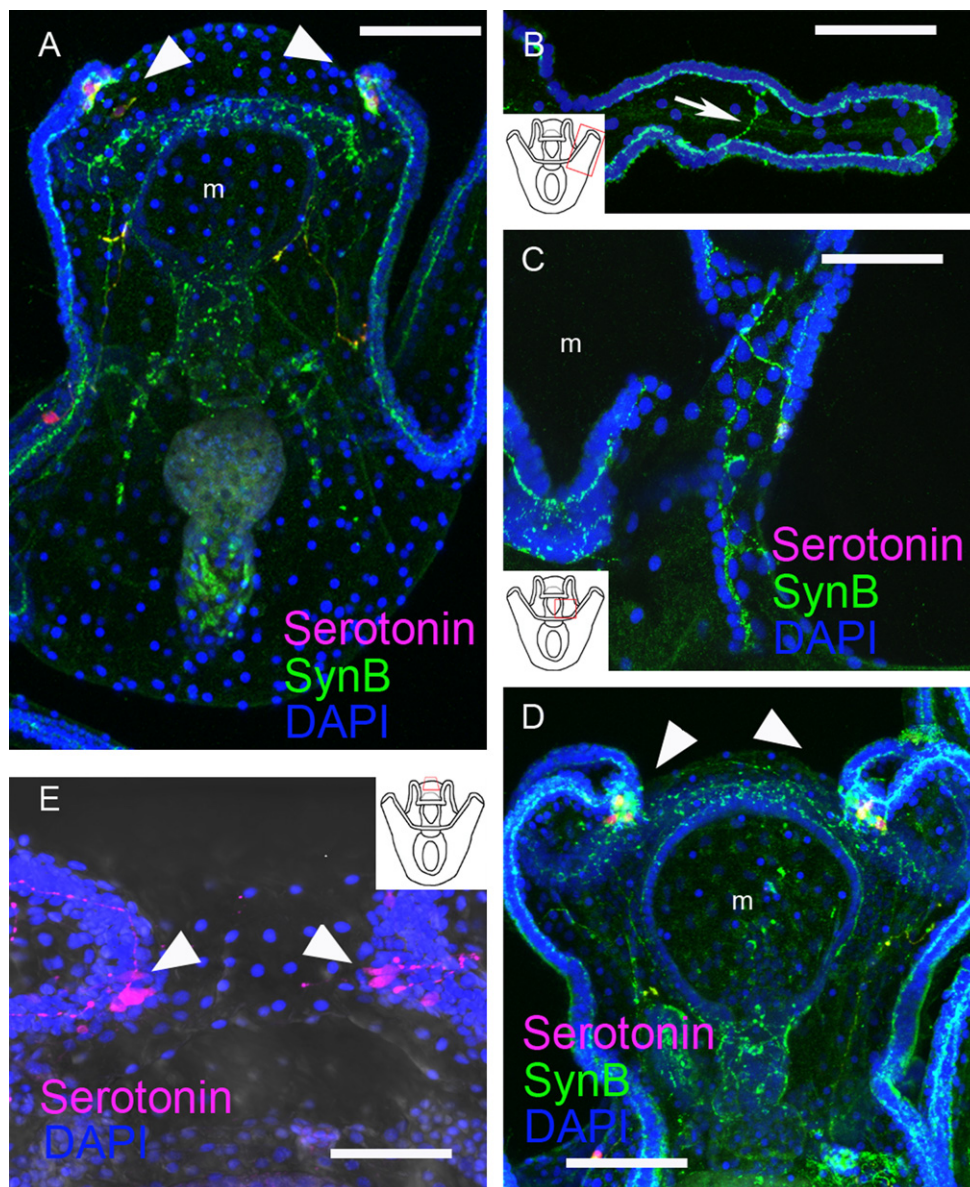
Fig. 4. Expression of synaptotagmin and serotonin in 7–13 day larvae. (A) Dorsal view of a 7-day larva. Serotonin containing neurons (arrowheads) are restricted to the pre-oral loops of the ciliary bands and larvae lack the discrete apical organ of euechinoids. (B) Arm of a 10-day larva showing a short axonal tract projecting beneath the oral ectoderm (arrow). (C) Larva (10 days) showing the two types of neurons commonly found in the ciliary bands: flask-shaped primary sensory cells and fusiform interneurons. (D,E) Details of the apical region of 13-day plutei. (D) There are a small number of serotonin containing neurons associated with the ciliary bands as the anterolateral arms form (arrowheads). (E) View of the apical end of the embryo showing the serotonergic cells (arrowheads) laterally placed between the preoral and developing anterolateral arms (composite DIC-confocal image). (A–D) Bars=20 μm, (E) Bar=10 μm
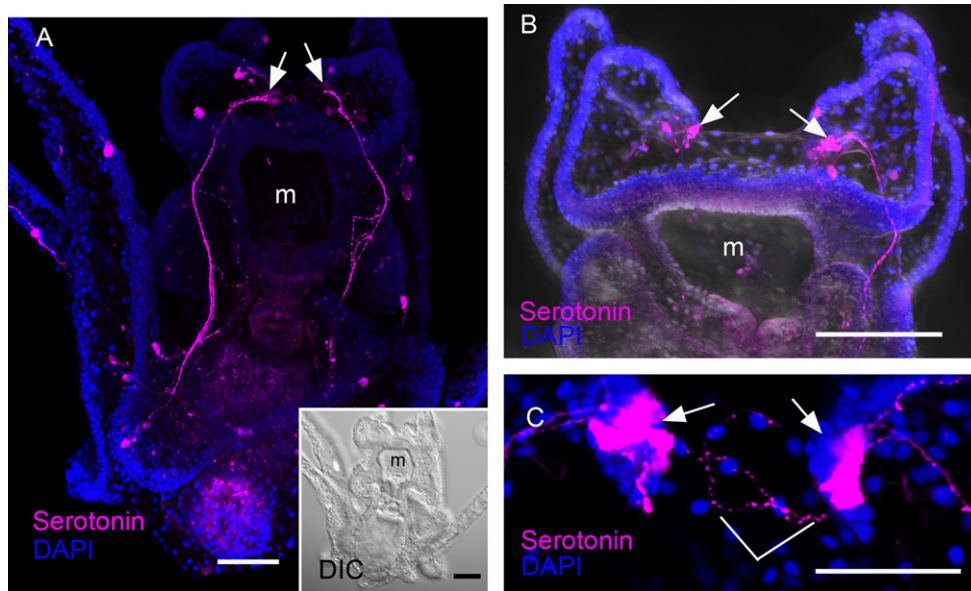
Fig. 5. Serotonergic neurons in 15-day plutei of *E. tribuloides*. (A) Serotonergic neurons in the ciliary bands connect to tracts of axons in the blastocoelar space, lateral to the gut, that connect to the serotonergic cells in the oral hood (arrows). Inset: DIC image of larva. (B) The two small clusters of serotonergic cells in the oral hood (arrows). (C) Detailed image of serotonergic neurons in the ciliary bands of the oral hood (arrows) showing the diminutive connection between them. A,B, inset. Bars=25 μm C. Bar=12 μm

the ciliary band tracts (Fig. 5A). Thus, the pluteus larva of *E. tribuloides* lacks a discrete apical organ with large clusters of neurons containing serotonin and synaptotagmin and a robust neuropil, features typical of euechinoid larvae.

The larval nervous system in *E. tribuloides* is similar to that of other echinoderm larvae in the distribution of neurons in the ciliary bands, esophagus, larval mouth and associated musculature. In a number of locations there are tracts of axons that leave the ciliary band and cross oral ectoderm, connecting to other portions of the ciliary bands (Fig. 4B and C). However, unlike other echinoplutei, there are no lateral ganglia between the post-oral and anterolateral arms with extensive projections of axons ramifying beneath the aboral ectoderm (cf. Nakajima et al., 2004a).

Discussion

The nervous systems of *E. tribuloides* plutei have several features similar to the nervous systems of all echinoid plutei. Neurons arise in association with the ciliary band and project axons that form tracts within the ciliary band. The initial ciliary band neurons are bipolar and project axons that surround the field of oral ectoderm. The ciliary band neurons develop into several morphological types, all of which are similar to the ciliary band neurons of euechinoids. In addition, the localizations of the ectodermal markers Hnf6 and Chd are similar to the localizations in euechinoids. In euechinoids, Hnf6 initially has a broad distribution but in plutei it serves as a marker for cells that will be part of the ciliary band (Poustka et al., 2004; Otim et al., 2004). In *E. tribuloides*, Hnf6 becomes restricted to the ciliary band cells and scattered cells in the oral ectoderm. The distribution of Chd suggests that the oral ectoderm is divided into sub-domains, as in euechinoids (Bradham et al., 2009; Saudemont et al., 2010). These features indicate that there are overall similarities in ectodermal and neural development among all echinoids.

Notwithstanding the similarities, the nervous system of *E. tribuloides* larvae has a number of features that distinguish it from larval nervous systems of euechinoids. Perhaps the most striking feature is the lack of a thickened animal plate with a tuft of non-motile cilia. The animal plate in euechinoids is a distinct

domain of ectoderm as several genes are expressed exclusively within this region (Burke et al., 2006; Poustka et al., 2007; Saudemont et al., 2010; Yaguchi et al., 2010b, 2012), of which several are critical to the development of the apical organ and innervation of the ciliary band (Wei et al., 2009; Yaguchi et al., 2008). However, the blastulae and gastrulae of asteroids, holothuroids and ophiuroids do not have an animal plate. In most feeding larval forms of echinoderms, the apical serotonergic neurons arise laterally in anterolateral ciliary band (Byrne et al., 2007). This pattern is seen in asteroids (Chee and Byrne, 1999; Yankura et al., 2010), holothuroids (Nakano et al., 2006; Bishop and Burke, 2007) and ophiuroids (Hirokawa et al., 2008; Dupont et al., 2009). It is noteworthy that in hemichordates (the sister taxon to the echinoderms) with a feeding larval form, serotonergic neurons also develop lateral to the animal pole in association with ciliary bands (Nakajima et al., 2004b; Miyamoto et al., 2010). Thus, the animal plate with distinctive non-motile ciliary tuft appears to be a derived feature of euechinoids and lateral origins to serotonergic cells and the absence of an animal plate is the pattern common to other ambulacrarians.

Although asteroid embryos do not have a thickened animal plate and ciliary tuft, they do express genes that are characteristic of this region in what is termed the animal pole domain (Yankura et al., 2010). Yankura et al. note that the domain is not morphologically distinct, but encompasses a broad region of ectoderm at the anterior end of the embryo. The pattern of expression of Hnf6 and Nk2.1 protein we observe in *E. tribuloides* is strikingly similar to the expression domains of orthologues of these genes reported for the bipinnaria. Yankura et al. report that in asteroid embryos and early larvae, Six3 is expressed throughout the broad animal pole domain, the mouth and esophagus (cf. Fig. 4; Yankura et al., 2010). However, were able to detect the protein only in a few cells in lateral region of pre-oral transverse and the lower lip of the mouth in *E. tribuloides*. A similar situation occurs in the euechinoid *S. purpuratus*, where Six3 mRNA accumulates in the animal plate, but only a few cells on the margins express the protein (Burke, unpublished). These differences suggest post-transcriptional regulation of Six3. Overall, the expression patterns of Nk2.1 and Hnf6 suggest that *E. tribuloides*, like other echinoderm larvae lacking an animal plate, has a broad animal pole domain. However, for a more

robust comparison, this point needs to be further investigated in *E. tribuloides* with a range of *in situ* hybridization probes.

Bennett et al. (2012) provide a detailed description of the embryonic and larval development of another cidaroid, *Cidaris blakei*. They note that a thickened animal plate with a tuft of non-motile cilia is present in early gastrulae of *C. blakei*. In addition, their review of descriptions of cidaroid development reveals that another species in the same genus is also reported to have an animal plate. Their analysis suggests that this feature has been lost in the four species reported to lack animal plates, whereas based on descriptions of other echinoderms, the most parsimonious interpretation is that animal plates and ciliary tufts are derived features. If the ciliary tuft of these species of *Cidaris* is identical to that of euechinoids, the expression of animal pole domain genes will be restricted to the animal plate region. The question bears further investigation as the gain or loss of this feature in several lineages suggests it is not as highly conserved as is often proposed (Dunn et al., 2007; Jackson et al., 2010).

Another remarkable feature of the larval nervous system of *E. tribuloides* is that it lacks the discrete apical organ of euechinoid larvae. The apical organ of *Psammechinus miliaris* is typical of euechinoids and in eight-arm plutei it has 25–50 serotonergic neurons in a bilaterally symmetric patch on the oral hood (Beer et al., 2001). At a similar stage, *E. tribuloides* has fewer than five serotonergic neurons associated with each pre-oral loop of the ciliary bands. In euechinoid apical organs there is a robust neuropil

underlying the neurons (Beer et al., 2001). In contrast, *E. tribuloides* larvae form a commissure consisting of a few individual axons. This pattern is more similar to the arrangement of serotonergic cells in asteroids, holothuroids or ophiuroids (Burke, 1983; Moss et al., 1994; Chee and Byrne, 1999; Nakajima et al., 2004a Nakano et al., 2006; Bishop and Burke, 2007; Hirokawa et al., 2008; Dupont et al., 2009; Cisternas and Byrne, 2003).

As larval forms of most metazoans have apical sensory organs with associated serotonergic cells, it has been suggested that apical organs are a derived metazoan feature (Jagersten, 1972; Reichert and Simeone, 2001; Denes et al., 2007). The patterns of expression of several genes and the details of development in diverse taxa identify a number of similar features of gene expression (Takacs et al., 2004; Jackson et al., 2010; Santagata et al., 2012; Arendt et al., 2008). However, distinguishing conservation of ancestral metazoan features from convergence based on similar mechanisms of neurogenesis has proven problematic. For instance, Dunn et al. (2007) assert that the apical organs of urchins and gastropods are specified by different gene regulatory networks and are thus a result of convergent evolution. Yet, it is clear that a similar set of genes including Six3, FoxQ2, Otp, Hbn, Fez, and Nk2.1 specify a domain at the animal pole of a wide range of metazoans (Santagata et al., 2012; Wei et al., 2009; Burke et al., 2006; Yankura et al., 2010). Our observation that the organization of the apical organ of *E. tribuloides* is more similar to that of other echinoderms than it is to euechinoids supports the hypothesis that the discrete

Table 1
Summary of Ambulacrarian Larval Neuroanatomy. References provided in text.

Feature	Hemichordate	Bipinnaria	Ophiuroid Pluteus	Auricularia	Eucidaroid Pluteus	Euechinoid Pluteus
Neurons and axon tracts embedded in the ciliary band surrounding the oral epidermis	Yes	Yes	Yes	Yes	Yes	Yes
Serotonergic Cells on the oral hood	Yes	Yes	Yes	Yes	Yes	Yes
Extensive neural projections beneath the oral epidermis	Yes	Yes	No	No	No	No
Thickened Animal Plate in gastrula	No	No	No	No	No	Yes
Apical organ separate from the ciliary bands on oral hood	No	No	No	No	No	Yes
Neurites project from lateral ganglia beneath the aboral epidermis	No	No	No	No	No	Yes
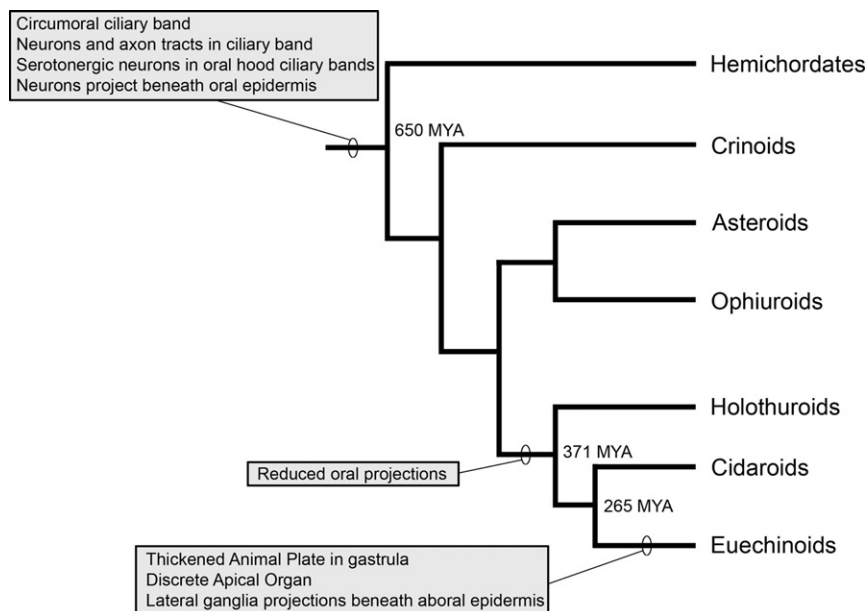
Fig. 6. Dendrogram depicting relationships of ambulacraria and echinoderm classes to the cidaroids and the euechinoids. The notes specify features of larval neuroanatomy that distinguish the branch. The estimated time of divergence are from Peterson et al. (2008), Wada and Satoh (1994), and Smith et al. (2006).

apical organ of euechinoids is derived. Yet, it seems clear that the expression of a common set of genes in a neurogenic animal pole domain is a conserved metazoan feature. Thus, the morphological manifestation of the underlying genetic network is labile – gastrulae with a thickened animal plate and ciliary tuft that gives rise to a separate ganglionic apical organ appears to have arisen several times in metazoan evolution. The details of the genetic and signaling networks operating at the anterior end of metazoan larvae remains the level at which to focus attention to determine what aspects of anterior sensory organ development are conserved in metazoans.

All euechinoid larval nervous systems investigated to date have a small cluster of neurons between the post-oral arms and the pre-oral or posterolateral arms, termed the lateral ganglion (Nakajima et al., 2004a). Lateral ganglia project numerous neurites posteriorly beneath the aboral ectoderm. This is an additional feature of euechinoid larval nervous system that appears to be lacking from plutei of *E. tribuloides*. However, because the function of these lateral ganglion neurons and the dendrites or axons that project from them are not known, the significance of this missing feature in *E. tribuloides* is not at all clear.

Patterning of ectoderm in euechinoids is a consequence of vegetal signaling (Angerer et al., 2000; Duboc et al., 2004; Lapraz et al., 2009; Saudemont et al., 2010). In euechinoids, vegetal signaling initiated by accumulation of β-catenin in nuclei directly establishes endomesoderm precursors and initiates signaling that establishes ectoderm domains and restricts neurogenesis to the anterior end of the embryo (Logan et al., 1999; Angerer et al., 2000; Duboc et al., 2004; Lapraz et al., 2009; Saudemont et al., 2010 Yaguchi et al., 2006; 2008; 2010a). In *S. purpuratus*, a complex mechanism of canonical and non-canonical wnt signaling restricts neurogenesis to the animal pole domain (Range et al., 2013). Differences in how this pathway functions may produce the morphological differences in neural development we observe in *E. tribuloides*. Lithium, zinc and 1-azakenpaullone (a GSK3 inhibitor, Kunick et al., 2004) treatments produce embryos that are similar in form to embryos in which specific aspects of vegetal signaling are blocked or enhanced (Lallier, 1975). The response of *E. tribuloides* embryos to interference with, or enhancement of the signaling that restricts neurogenesis to the anterior pole suggests a similar mechanism operates in all echinoids, in spite of the lack of a discrete apical organ (Supplementary Fig. S1). Thus, changes in the mechanisms restricting and patterning neurogenesis are clearly a potential mechanism by which neuroanatomical novelty arose in euechinoids. These observations enable an experimental investigation of the mechanisms that underlie the differences in neuroanatomy and causes of evolutionary change in the broadly conserved developmental programs that regulate embryonic neurogenesis.

We conclude that the larval nervous system of *E. tribuloides* has features shared with other classes of echinoderms, but not with euechinoids (Table 1 and Fig. 6). It is apparent from our data that a thickened animal plate, ciliary tuft, a discrete apical organ, and lateral ganglia with axons projecting posteriorly are derived feature of euechinoids. The concordance of the fossil record and genetic sequence analysis indicates the time of radiation of extant echinoids can be determined (Kroh and Smith, 2010). Assuming that features of extant cidaroids represents the ancestral echinoid state, our analysis of neural development and larval nervous system structure in *E. tribuloides* allows the conclusion that the shared specialized features of larval nervous system of euechinoids arose after the divergence and radiation of euechinoids, about 265 MYA. Establishing an evolutionary history for developmental changes in the organization of the sea urchin larval nervous systems enhances the usefulness of this model of deuterostome evolution and development.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.03.006.

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