

Peptidergic and serotonergic immunoreactivity in the metamorphosing ophiopluteus of *Ophiactis resiliens* (Echinodermata, Ophiuroidea)

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Abstract. Antibodies against the echinoderm-specific neuropeptide S1 and against 5HT were used to examine the fate of the larval nervous system during metamorphosis in the ophiuroid *Ophiactis resiliens*. In contrast to most echinoderms, the onset of peptidergic and serotonergic expression was delayed to the advanced ophiopluteus stage, in particular for 5HT. In advanced ophioplutei, peptidergic immunoreactivity was located in simple fibres associated with the ciliated bands, a stomach nerve ring, and cells along the antero-lateral arms. 5HT immunoreactivity was concentrated in 2 oral ganglia in the adoral projections, located at the posterior rim of the mouth. Clusters of 5HT-positive cells were also found along the antero-lateral arms. The ophiopluteus lacked a serotonergic (or peptidergic) anterior ganglion. In echinoids, holothuroids, and crinoids, anterior ganglia are thought to have a sensory role in settlement and metamorphosis. Given that ophioplutei metamorphose in the plankton and that larval structures degenerate before settlement, the absence of apical ganglia correlates with the lack of a functional role for larval structures in substrate selection and settlement. Although most of the larval nervous system degenerated during metamorphosis, the adoral projections and associated oral ganglia appeared to be incorporated into the juvenile mouth, suggesting a potential role for larval neurons in contributing to oral neuronal structures in the adult. S1-positive neurons and fibres in the rudiment developed *de novo* and in parallel with development of the epineural canal. This structure gives rise to the primordia of the adult circumoral nerve ring and radial nerves, indicating that differentiation of the adult nervous system begins in the early stages of metamorphosis.

Additional key words: larval nervous system, neuropeptides

Histological and histochemical studies have provided great insights into the organization and structure of the larval nervous system of many echinoderms. The planktotrophic larvae of echinoids, holothuroids, and asteroids have a network of cells and fibres, primarily associated with the ciliated bands and the digestive tract (Chen et al. 1995; Beer et al. 2001; Byrne et al. 2001). In particular, antiserum to the neuropeptide GNSALMFamide-1 (S1), first isolated from an asteroid, binds to larval neurons in echinoids, asteroids, and ophiuroids, making it an ideal neuronal marker for comparative studies of neurogenesis across taxa (Bisgrove & Burke 1986; Elphick et al. 1991a; Thorndyke et al. 1992; Cisternas et al. 2001). The prevalence of serotonin (5HT) in the larval nervous systems of echinoids and asteroids and in larvae of most marine invertebrates suggests that this neurotransmitter also

plays an important role in larval development (Barlow & Truman 1991; Hay-Schmidt 2000; Beer et al. 2001; Chee 2001). Many larvae have serotonergic apical ganglia, which are suggested to have a sensory role in settlement and metamorphosis (Hay-Schmidt 2000). Although functional studies of the larval nervous system are not yet available, the prevalence of such neurotransmitters in the ciliated bands and the digestive tract has been taken to suggest a role in co-ordination of feeding and swimming (Bisgrove & Burke 1987; Thorndyke et al. 1992; Ghyoot et al. 1994; Moss et al. 1994; Debremaeker et al. 1997; Byrne et al. 2001).

The organization of the adult nervous system of echinoids, asteroids, and ophiuroids has also been documented through ultrastructural and immunocytochemical studies (Elphick et al. 1991a,b; Debremaeker et al. 1997; Byrne et al. 1999; Byrne & Cisternas 2002). These studies have shown that S1 is prevalent in both adult and larval nervous systems. In most echinoderms, however, little is known about the relation-

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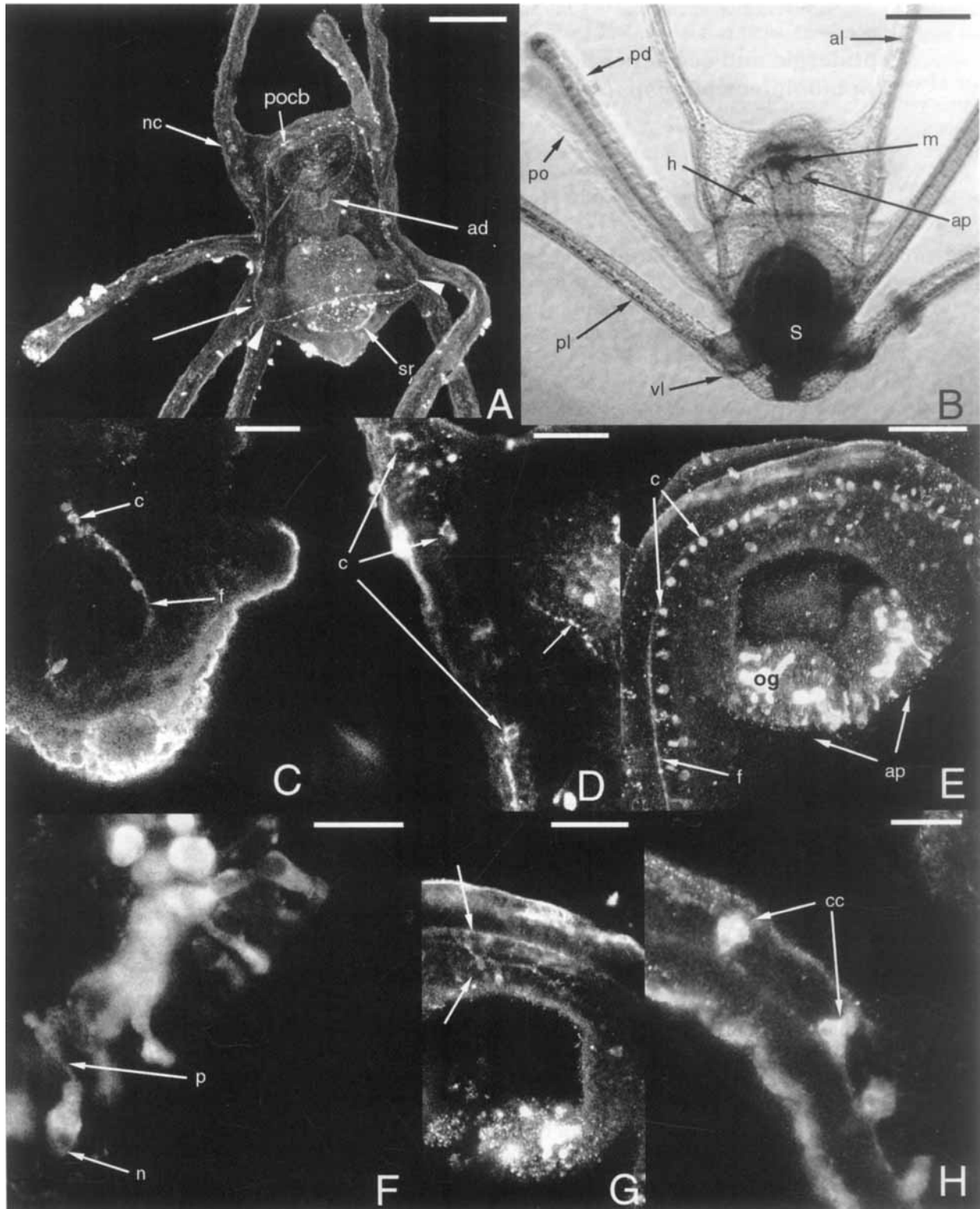


Fig. 1. Advanced 8-armed ophioplutei of *O. resiliens*. Confocal (A, C–H) and light (B) micrographs. **A.** S1-IR in the adoral ciliated band (ad), the stomach ring (sr) and along the ciliated band (arrow). Nerve cell (nc), pre-oral ciliated band (pocb). Scale bar, 100 μ m. **B.** 90-day-old larva with hydrocoel (h), vibratile lobes (vl), and adoral projections (ap). Antero-lateral arm (al), post-oral arm (po), postero-dorsal arm (pd), postero-lateral arm (pl), mouth (m), stomach (S). Scale bar, 100 μ m. **C.** Stomach region of an early metamorphosing ophiopluteus showing scattered S1-IR nerve cells (c) and fibres (f). Scale

ship between the larval and adult nervous system during metamorphosis. During this period, the adult body and nervous system appear to develop *de novo*, but the extent to which the larval body contributes to the juvenile body varies considerably between echinoderm classes (Mooi & David 1998; Mooi 2001). E.g., in echinoids, the juvenile body axis develops independently of the larval body, whereas, in holothuroids and ophiuroids, the larval body axis and some larval structures are retained in the juvenile (Chia & Burke 1978; Smiley 1986; Burke 1987). In this study, we investigated the fate of the larval nervous system of the ophiuroid *Ophiactis resiliens* during metamorphosis. Neuronal markers to S1 and 5HT were used to document spatial and temporal changes in peptidergic and serotonergic neuronal expression in advanced and metamorphosing larvae to determine whether parts of the larval nervous system are incorporated into the adult nervous system.

Methods

Mature specimens of the brittle star *Ophiactis resiliens* LYMAN 1879 were collected near Sydney, Australia, during June–August 1999. Spawning was induced by light/dark-temperature shock treatment (Selvakumaraswamy & Byrne 2000). Fertilized eggs were rinsed in 1- μm filtered seawater (FSW) and cultures were set up at densities of 2500 eggs/L. Larvae were reared at 19–24°C and fed $\sim 2 \times 10^4$ cells/ml of *Chaetoceros calcitrans* (CS-178, CSIRO Marine Laboratories). Cultures were gently stirred with paddles, and the FSW was replaced every 4 days.

Advanced 8-armed ophioplutei and metamorphosing larvae (30–99 d old) were processed for immunocytochemistry; 10–20 larvae from each of 5 cultures were fixed in 2% paraformaldehyde in FSW, for 16 h at 4°C. Fixed larvae were rinsed in phosphate buffer saline (PBS), 3×10 min to remove excess fixative and salts. Larvae were then placed in blocking solution, consisting of 5% goat serum plus 0.3% Triton-X, diluted in PBS containing 0.1% bovine serum albumen (BSA) for 1 h at room temperature. After 3 rinses in PBS, larvae were incubated in primary antibody diluted in 0.1% BSA in PBS, for 16 h at 4°C. Primary antibodies used were: (a) rabbit anti-GNSALMFamide-1

serum (S1) diluted 1:2000 and (b) rabbit anti-5HT (Sigma), diluted 1:200. Larvae were rinsed in PBS (3×10 min) and incubated in goat anti-rabbit-Texas red conjugated secondary antibody, diluted 1:500 in PBS, for 1.5 h in the dark, at room temperature. Finally, larvae were rinsed in PBS buffer (3×10 min) and mounted on slides with Vectashield® anti-fade medium. Coverslips were sealed with nail polish.

Controls for immunocytochemistry of larvae included staining (1) in the absence of primary antibody, (2) in the absence of secondary (goat) antibody, and (3) with pre-immune rabbit serum (Sigma) replacing primary antibody. No IR was observed in these controls.

Larvae were initially examined under epifluorescence (596 nm excitation) to locate immunoreactive sites. Serial images of individual larvae were then produced on an MRC600 confocal laser-scanning microscope (Biorad®) and 3-D reconstructions were created, using Confocal Assistant v4.02 (Biorad®), to determine the position of immunoreactive structures within the larval body. Bright-field images of the larvae also assisted with interpretation of 3-D reconstructions.

Results

Advanced 8-armed ophioplutei

The ophioplutei of *Ophiactis resiliens* reached the early 2-armed stage ~ 48 h after fertilization. S1-immunoreactivity (S1-IR) first appeared in the form of a nerve encircling the stomach at the 6-armed stage (results not shown here). This nerve consisted of several cell bodies (7–10 μm in diam.) connected by fine nerve fibres that were dotted with varicosities. The 8-armed ophiopluteus developed at ~ 30 d after fertilization and the nerve around the stomach increased in complexity. These ophioplutei had additional S1-IR fibres in the epithelium of the ciliated band. The ciliated band bordering the oral hood (pre-oral ciliated band) and the post-oral transverse band between the post-oral arms were innervated by a S1-IR fibre characterised by numerous varicosities. The pre-oral and post-oral ciliated bands were connected by an IR fibre that extended laterally along the oral region (Fig. 1A). An S1-IR fibre also innervated the adoral ciliated band along the lower rim of the mouth opening (Fig. 1A,D).

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bar, 50 μm . **D.** Base of right antero-lateral arms showing small clusters of S1-IR cells (c). Dotted S1-IR corresponds to fibre along the mouth opening (arrow). Scale bar, 20 μm . **E.** 5HT-IR cells (c) and connecting fibre (f) along the oral hood and bilateral oral ganglia (og) in the adoral projections (ap) of the mouth. Scale bar, 20 μm . **F.** Left oral ganglion showing sensory-like 5HT-IR cells, with a small basal nucleus (n) and apical process (p). Scale bar, 10 μm . **G.** Small network of 5HT-IR fibres and scattered cells located in the oral hood (arrows). Scale bar, 10 μm . **H.** 5HT-IR in groups of 2 or 3 cells (cc) and adjoining fibre along the left antero-lateral arm. Scale bar, 10 μm .

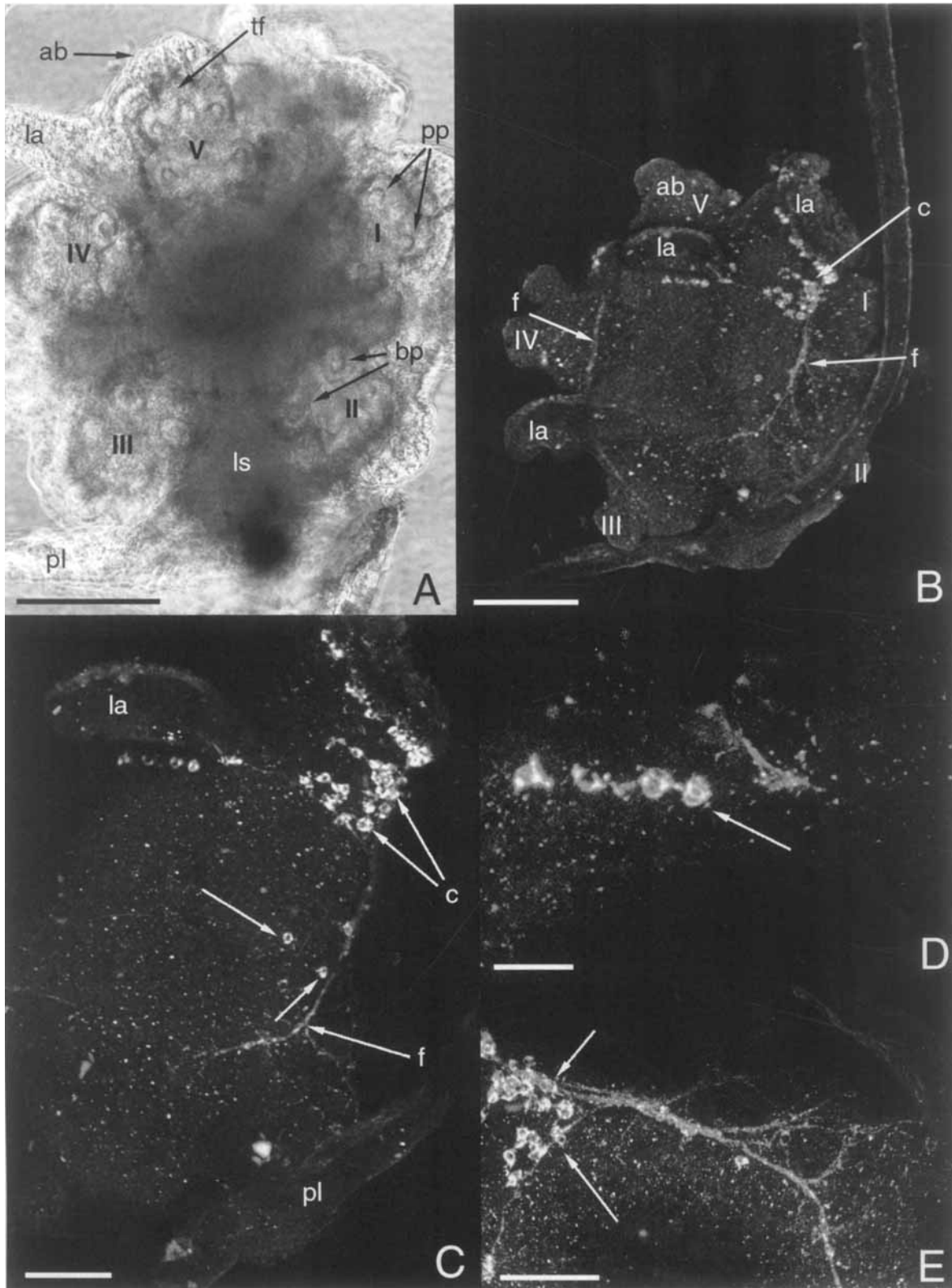


Fig. 2. S1-IR in metamorphosing ophioplutei of *O. resiliens*. Light photomicrograph (A) and confocal reconstructions (B–E). **A.** 97-day-old larva showing radial juvenile, with developing arm buds (ab) I–V, terminal tube foot (tf), primary podia (pp), buccal podia (bp), larval arms (la) in the process of being resorbed, and degenerating larval stomach (ls). Postero-lateral arm (pl). Scale bar, 100 μ m. **B.** 99-day-old metamorphosing larva showing S1-IR in a group of cells (c) in the developing rudiment and between juvenile arm buds I and V. S1-positive fibres (f) form a partial ring in the rudiment.

A cluster of 2 or 3 S1-IR cell bodies appeared at the base of the antero-lateral arms (Fig. 1A). At this stage, 5HT-immunoreactivity (5HT-IR) was also observed in 1 or 2 cells in the adoral region.

Advanced ophioplutei (90 d) had a 5-lobed hydrocoel and 2 vibratile lobes, extensions of the ciliated band located at the base of the postero-lateral arms (Fig. 1B). S1-IR was similar to that in earlier 8-armed stages. The S1-IR nerve around the stomach had shifted to the right, in parallel with morphogenetic movements of the stomach. The S1-IR fibres of the stomach were weakly immunoreactive and few cell bodies (2 or 3) were present (Fig. 1C). This decrease in IR coincided with degeneration of the larval gut during metamorphosis. Portions of the S1-IR fibre associated with the pre- and post-oral ciliated bands, and their interconnecting fibre, also degenerated. However, a new group of S1-IR cells with a connecting fibre appeared along the proximal end of the antero-lateral arms in these advanced larvae (Fig. 1D).

Advanced ophioplutei were also characterised by a concentration of 5HT-IR in the anterior region of the larvae. Two (adoral) projections developed as evaginations of the buccal floor; these may play a role in opening and closing the larval mouth (Selvakumaraswamy 2002), and they were bordered by the adoral ciliated band (Fig. 1B). In each projection, ~20–30 cells formed a pair of ganglia and showed 5HT-IR (Fig. 1E). The cells in these oral ganglia were monopolar and flask-shaped (~10 µm diam.) with a basal nucleus and a narrow apical region that gave rise to a short cilium-like process (~7 µm, $n = 3$) that projected towards the oral cavity (Fig. 1E,F). In the oral hood, large round 5HT-IR cell bodies (5–6 µm diam.), connected by a thin IR fibre, were observed (Fig. 1E). Diffuse 5HT-IR fibres and cells appeared to innervate a small region of the oral hood and the roof of the oral cavity (Fig. 1G). Clusters of 3 or 4 5HT-IR cells, connected by a thin IR fibre, were evenly spaced along each antero-lateral arm (Fig. 1H). No similar structures were present in other larval arms. The location of these 5HT-IR cells coincided with cells also labeled by the antibody against S1 at the base of the antero-lateral arms.

Metamorphosing larvae

In late metamorphosing larvae (97 d old), the hydrocoel attained its pentamerous form and the primary

tube feet, including the terminal tube foot and buccal podia, had developed (Fig. 2A). By this stage, the larval stomach and arms, except for the postero-laterals, were being resorbed. As a result, most of the S1-IR seen in the stomach and ciliated band had degenerated. Similarly, there was no trace of serotonergic structures and no further 5HT-IR was observed in the later stages of metamorphosis.

In metamorphosing larvae (98–100 d old), S1-IR was largely restricted to diffuse fibres located within the developing rudiment. These fibres, dotted with varicosities, extended around the base of the developing arm buds (Fig. 2B,C). S1-IR cells and fibres associated with the pre- and post-oral ciliated band regions and the stomach disappeared as the larval body was resorbed. An aggregation of S1-IR cell bodies appeared in the rudiment at the base of the antero-lateral arms, which were in the process of being resorbed (Fig. 2C,D). The region between the antero-lateral arms contained the larval mouth, as well as the adoral projections, which were displaced toward the oral side of the developing juvenile. The adoral projections were later incorporated into the juvenile mouth whereas the remainder of the pre-oral lobe of the larvae was resorbed. Interconnecting fibres that encircled the rudiment appeared to innervate cells at the site where the antero-lateral arms were being resorbed (Fig. 2C), that is between arm buds I and V (following the numbering system of Olsen (1942) whereby arm buds are identified from developing hydrocoel lobes). Developing arm buds I and II were also innervated by branching S1-IR processes (Fig. 2E). It is not clear if these cells and fibres were associated with the developing adult structures or were part of the larval nervous system. Due to the opacity of the rudiment, it was not possible to resolve fibres and cells deeper inside the rudiment with confocal microscopy. A row of spindle-shaped cells (~7 µm long) and developing fibres were also observed in other areas of the rudiment (Fig. 2C,D).

Discussion

Larval nervous system

Like the planktotrophic larvae of other echinoderms, the feeding larvae of *Ophiactis resiliens* had a well-developed nervous system. Peptidergic and serotonergic immunoreactivity (IR) similar to that seen here has been reported for echinopluteus, bipinnaria,

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Scale bar, 100 µm. **C.** Detail of larvae in B showing S1-positive fibres (f) and cells (arrows) in the rudiment and in a cell cluster (c). Scale bar, 50 µm. **D.** Row of S1-positive cells in the rudiment (arrow). Scale bar, 20 µm. **E.** Details of S1-IR fibres developing in the rudiment between arm buds I and II (arrows), as shown in B. Scale bar, 50 µm.

brachiolaria, and auricularia larvae (Chen et al. 1995; Beer et al. 2001; Byrne & Cisternas 2002). This and previous investigations on the larvae of *O. resiliens* (Cisternas et al. 2001) appear to be the first immunocytochemical studies of the ophiuroid larval nervous system. Previously, a histochemical study revealed the presence of catecholamines in the ciliated band in the ophioplutei of *Ophiothrix fragilis* (Cottrell & Pentreath 1977).

The distribution of peptidergic and serotonergic IR in the ophioplutei of *Ophiactis resiliens* was predominantly associated with the ciliated band and the oral region. The position of the S1-IR fibre connecting the pre- and post-oral ciliated bands of *O. resiliens* corresponds to that of a row of cells (or nuclei) first described in other ophioplutei by Mortensen (1921, 1931) and the catecholergic lateral nerve tracts joining the pre- and post-oral ciliated bands in the auricularia (Burke et al. 1986; Chen et al. 1995). This suggests that the peptidergic innervations of the oral region may be involved with co-ordination of ciliary activity of the pre- and post-oral ciliated bands. Peptidergic IR in this ophiuroid resembled the pattern shown in echinoplutei with another SALMFamide antibody, S2 (Beer et al. 1998), potentially reflecting the strong convergence in larval form between echinoids and ophiuroids. The peptidergic and serotonergic systems in *O. resiliens* appeared to overlap in cells associated with the antero-lateral arms. Co-localization studies are required to confirm that the same population of cells are involved. Serotonergic and dopaminergic cells and axonal tracts along the ciliated band of the larval arms in echinoplutei are suggested to have sensory function (Bisgrove & Burke 1987; Beer et al. 1998). In contrast, peptidergic and serotonergic IR is absent in the early larvae of *O. resiliens*. This suggests that other neurochemicals may be associated with early development. Given that a number of neuropeptides have been characterised from other echinoderms (Elphick et al. 1991b; Diaz-Miranda et al. 1992), the potential role and localization of neuropeptides in ophiuroid larvae may not be resolved until the putative ophiuroid S1 equivalent is identified. Pre-neuronal expression of serotonin during gastrulation is characteristic of asteroid and echinoid development (Bisgrove & Burke 1987; Nakajima 1988; Thorndyke et al. 1992; Moss et al. 1994; Beer et al. 1998; Chee & Byrne 1999a) but was not observed in *O. resiliens* (Cisternas et al. 2001).

The major feature of the nervous system in advanced ophioplutei was the serotonergic oral ganglia in the adoral projections. Serotonergic ganglia associated with the lower rim of the mouth opening and adoral ciliated band are prevalent in echinoderms and

have been reported in echinopluteus, auricularia, and brachiolaria larvae (Burke 1983a,b,c; Burke et al. 1986; Bisgrove & Burke 1987; Chen et al. 1995; Chee & Byrne 1999a,b; Beer et al. 2001). In all these larvae, the oral ganglia are bilateral structures as in *O. resiliens*. In echinoids and asteroids, however, anastomosing axonal tracts associated with these ganglia innervate the esophagus (Burke et al. 1986; Thorndyke et al. 1992; Chen et al. 1995; Chee 2001). No such anastomosing network was observed in *O. resiliens*. In asteroids, peptidergic neurons and fibres in the oral region appear soon after the onset of feeding and develop into extensive neuronal networks associated with feeding structures (Thorndyke et al. 1992; Moss et al. 1994; Beer et al. 1998; Chee & Byrne 1999a; Byrne & Cisternas 2002). This pattern of expression has been taken to suggest that IR cells may be involved with feeding behaviour (Moss et al. 1994; Beer et al. 1998; Byrne et al. 2001; Byrne & Cisternas 2002). 5HT-IR in the oral ganglia of echinoplutei is delayed to the 6–8 armed stages, when the rudiment starts to form, although catecholaminergic and peptidergic neurons appear around the onset of feeding (Bisgrove & Burke 1987; Thorndyke et al. 1992; Beer et al. 1998). The appearance of serotonergic neurons in the oral ganglia of *O. resiliens* parallels the temporal expression of this neuropeptide in echinoplutei and suggests that the oral ganglia in ophioplutei may play a role related to metamorphosis rather than feeding. Perhaps the oral ganglia are involved with detection of the exogenous cues required to complete metamorphosis. The oral region has been shown to be an important stimulatory centre for the induction of metamorphosis in *Dendraster excentricus* (Burke 1983a).

Unlike echinoid, holothuroid, asteroid, and crinoid larvae (Burke et al. 1986; Chia et al. 1986; Bisgrove & Burke 1987; Thorndyke et al. 1992; Nakajima et al. 1993; Chen et al. 1995; Beer et al. 1998), the larva of *O. resiliens* displayed no serotonergic/peptidergic ganglia in the anterior-most region. If anterior (= apical) ganglia were present in our ophioplutei, they were not 5HT or S1 positive. Immunocytochemical studies that target a wider range of ophiuroid species are required to determine if this pattern is typical of ophiuroids. Although it could be argued that a different suite of neurotransmitters or neuropeptides may identify apical ganglia in ophioplutei, no apical ganglia were evident by catecholaminergic activity in *O. fragilis* (Cottrell & Pentreath 1977).

Traditionally, the anterior larval region and apical ganglia are thought to play an important sensory role in detection of exogenous cues for induction of settlement and metamorphosis in echinoderm larvae. In echinoplutei, the apical ganglia contain peptidergic

and serotonergic cells that innervate structures involved in substrate selection and settlement (Burke 1983b,c; Bisgrove & Burke 1987; Thorndyke et al. 1992; Beer et al. 1998, 2001). Similarly, a serotonergic and catecholaminergic apical ganglion which develops in the anterior region of holothuroid larvae is also involved in substrate selection (Burke et al. 1986; Chen et al. 1995). In the advanced brachiolaria, peptidergic ganglia innervate the brachiolar complex and serotonergic cells develop in this attachment structure (Moss et al. 1994; Beer et al. 1998; Chee & Byrne 1999b; Byrne et al. 2001; Byrne & Cisternas 2002). Although the presence of neurotransmitters has not been examined in the apical organ that is associated with substrate selection in crinoid larvae, neuronal cells with the morphology of sensory cells have been described (Chia et al. 1986). In contrast to these echinoderms, planktotrophic ophiuroids metamorphose in mid-water, and substrate selection takes place after larval structures have degenerated. Substrate selection and attachment are executed by the juvenile tube feet. Thus, if the larval apical ganglia function primarily in settlement, the absence of these structures in the ophiopluteus of *O. resiliens* can be related to the fact that the ophiuroid larval nervous system is not involved in substrate selection. Differences between ophioplutei and other echinoderm larvae in the pattern of metamorphosis and settlement may account for the absence of apical ganglia in ophiuroids.

Metamorphosis

Most of the IR seen in the ophioplutei of *O. resiliens* disappeared during metamorphosis as larval structures were resorbed. The disappearance of 5HT-IR at metamorphosis is consistent with that reported for other echinoderm larvae and with the absence of this chemical in adult echinoderms (Cottrell & Pentreath 1970). By contrast, the prevalence of peptidergic cells in metamorphosing larvae suggests that some larval neurons may be incorporated into the developing rudiment. However, the origin of these cells could not be resolved in this study because their location overlapped both larval and adult structures. This cell cluster was positioned between arm buds I and V of the developing juvenile and coincided with the region where the larval mouth and the antero-lateral arms were being resorbed. Accordingly, these cells may correspond to peptidergic cells from either the antero-lateral larval arms or those associated with the oral ganglia that were undergoing a morphogenetic reorganization during metamorphosis. The adoral projections become incorporated into the adult mouth (McBride 1907; Selvakumaraswamy 2002), suggesting

a role for the oral ganglia in metamorphosis, perhaps as founder neurons for the adult oral nervous system. Alternatively, this cell cluster may represent newly differentiated adult neurons. Details of the differentiation of larval and adult neurons during metamorphosis will be essential to determine the origin of adult neuronal cells and the extent to which larval neurons (in particular, the oral ganglia) may contribute to the juvenile nervous system.

Peptidergic cells scattered in other regions of the rudiment may correspond to differentiating nerve primordia that give rise to the adult nerve ring and which appear at this stage of development in other ophiuroid larvae (McBride 1907; Narasimhamurti 1933; Fell 1945). In sections of the larvae of *Ophiocomina nigra*, *Ophiothrix fragilis*, and *Amphipholis squamata*, the primordium of the epineural canal first appears as fibres and large sets of nuclei extending from its walls (McBride 1907; Narasimhamurti 1933; Fell 1945). Concurrently, the radial nerve cord develops as cell aggregations and radiating fibres in each arm bud (McBride 1907; Narasimhamurti 1933; Fell 1945). In *O. resiliens*, numerous fibres appeared around arm buds V–II (i.e., V-I-II), suggesting that the radial nerve could be differentiating at this stage. The radial nerve cord of adult ophiuroids is strongly immunoreactive to S1 (Ghyoot et al. 1994; Debremaecker et al. 1997). Similarly, diffuse peptidergic IR has been reported in the circumoral and radial nerve cords in metamorphosing echinoplutei of *Psammechinus miliaris* (Beer et al. 2001). These findings indicate that differentiation of the adult nervous system starts in the early stages of metamorphosis.

Although expression of S1-IR was evident throughout development in *O. resiliens*, no clear demarcation between the adult and larval nervous systems was evident during metamorphosis, as adult nerve primordia appeared soon after the hydrocoel had fused and before all larval structures had degenerated. However, the persistence of peptidergic neurons at the site of the larval mouth suggests that some larval neurons may be incorporated into the developing adult nervous system. Detailed studies of ophioplutei during the metamorphic period, especially on the fate of the oral region, may provide interesting insights into the contribution of larval structures and the origins of the juvenile circumoral nerve ring.

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