# Neural Development in the Larva of Harmothöe imbricata (Linné): (Polychaeta; Polynoidae).

by

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## Summary

The nervous system of the trochophore larva of Harmothöe imbricata (Linné), (Polychaete, Polynoidae) includes larval nerves as well as rudiments of the adult nervous system. The appearance of neural tissue follows a developmental sequence beginning between the 6th and 7th day and correlates with behavioural development in the larva. It appears that early cerebral ganglion cells, including a pair of rhabdomeric ocelli, give rise to a prototrochal nerve that innervates prototrochal ciliated cells as well as muscle cells ensheathing and serving the stomodaeum. Three populations of vesicles/granules and three types of nerve fibers have been seen. One population of small, lucent vesicles is involved in synapse-like specializations. Another population resembles the known morphology of catecholaminergic granules. Neutral red staining and glyoxylic acid histochemical fluorescence confirm in part the involvement and distribution of catecholamines seen in the ultrastructural studies.

## Résumé

Le système nerveux de la larve trocophore de Harmothöe imbricata (Linné), (Polychaete, Polynoidae) comprend, en plus de neufs larvaires, les rudiments du système nerveux de l'adulte. L'apparition du tissu nerveux suit une séquence de développement débutant entre le 6ieme et 7ieme jour et cette séquence corespond au développement du répertoire comportemental de la larve. Il semblerait que les cellules primordiales du ganglion cérébral, comprenant une paire d'ocelles rhabdomériques, donnent naissance à un neuf prototrochal innervant les cellules ciliées du prototroch ainsi que les cellules musculaires enveloppant et servant le stomadéum. Trois populations de vésicules-granules et 3 types de fibres nerveuses ont été observées. Une population de petites vésicules claires est impliquée dans des spécialisations de type synaptique. Une autre population possède une morphologie ressemblant à ce qui est connu des granules catécholaminergiques. La coloration au rouge neutre et la fluorescence histochimique à l'acide glyoxylique confirment en partie le rôle et la distribution des catécholamines observés dans les études au niveau de l'ultrastructure.

## Acknowledgements

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To My Parents

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## Introduction

Harmothöe imbricata (Linné), (Polynoidae) is a subtidal, demersal marine polychaete found in the north temperate zone of both Europe (Thorson, 1946) and North America (Lacalli, 1980). The animals used in this study came from Passamaquoddy Bay, New Brunswick. The species is polytelic with an annual spring breeding season. It has been shown by Daly (1972) that females that are at least one year old develop two successive batches of oocytes in each season. Beginning in December, the maturation process of the first batch of oocytes continues until spawning in March. Immediately afterwards, the process is repeated for a second batch of ova that are spawned in late April. No more eggs are developed until the females prepare for spawning in the following year.

The structure and organization of the ovaries varies among polychaetes. Generally, oocytes are released from the gonad singly or in clusters after the premeitotic phase (Mill, 1978). In some nereids (Fischer, 1974, 1975) and a phyllodocid (Olive, 1974, 1975a,b), clusters of shed oogonia float independently in the coelom during the final stages of maturation. In Harmothöe imbricata (Daly, 1972), however, ovulation following vitellogenesis occurs immediately before spawning. There is cytological evidence in H. imbricata (Daly, 1974) that the ovary retains a proliferative stem cell or gametogonial population. In male H. imbricata the annual cycle of mitotic proliferation includes a phase of exponential growth followed by a time of steady state activity in the testes: experimental removal of spermatocytes causes a compensatory rise in proliferative activity in

the testes (Daly, 1974). In H. imbricata as well as many other polychaetes, functional genital ducts are lacking until sexual maturity when the coelomoducts and associated glands hypertrophy and acquire openings to the exterior to allow spawning to proceed; reversal of these changes may occur after spawning (Daly, 1972).

Preliminary unpublished experiments by Daly (Mill, 1978) suggest that a cerebral hormone is involved in vitellogenesis in H. imbricata. Temperature change as a specific environmental signal for gametogenesis appears to be significant. According to unpublished reports by Garwood (Mill, 1978), low temperature accelerates entry of developing oocytes into the growth phase while high temperatures prevent germ cell proliferation. Experimental manipulation of day length does not affect the early stages of gametogenesis in H. imbricata although moderately long day lengths increased oocyte growth and extremely long day lengths inhibited vitellogenesis.

Pair formation for breeding is characteristic of Harmothöe imbricata and has been studied by Daly (1972, 1973). During the two month breeding season the worms discontinue their solitary existance and form heterosexual pairs. At other seasons the worms avoid contact or fight when they encounter each other. At breeding, only mature males are permitted to mount the ripe female's dorsal surface. Between the female's parapodia, the elongated genital papillae of the male hang down to fertilize the released eggs. Fertilized eggs are trapped beneath the elytra until hatching (at 14 days according to Holborow and Laverack, 1972). The whole population does not spawn in synchrony during the breeding season.

Lacalli (1981b), in a study of the larvae found in plankton in Passamaquoddy Bay (New Brunswick) found feeding trochophores of H. imbricata in increasing numbers from late March until late April. The development of colour in the gut was coincident with an increase in diatom number. H. imbricata larvae are of typical polynoid shape and the body is mainly occupied by the stomach. The rapidity of swimming decreases as the larva enlarges and the body is not very muscular. Metamorphosing stages were found to be about 700 µm long with eight segments and simple setae (Lacalli, 1980).

Because of the complex nature of nervous systems in general, it is important and useful to study organisms with simple nervous systems and limited behavioural repertoires, requirements which are well met by many invertebrates. Recent work in the area includes nerve cell lineage studies in invertebrates such as in the leech, Helobdella triserialis (Weisblat, et al., 1978), a nematode worm, Caenorhabditis elegans (White, et al., 1978), and a snail, Aplysia californica (Kandel, et al., 1980). Pharmacological and electrophysiological studies on larvae are however, rare; pioneer work being the account by Carter (1926) on the nervous control of the velar cilia of the nudibranch veliger, a larval form studied again with more elaborate techniques by Mackie, et al., in 1976. Velar neurites were found to contain dense-core vesicles (Mackie, et al., 1976). But the identity of the normal mediator of ciliary arrests in gastropods larvae is still unknown. With the use of glyoxylic acid-induced fluorescence of catecholaminergic cells, the nervous system associated with the ciliary band of an echinoderm gastrula (Dendraster excentricus), was

shown to derive in part from animal plate cells and to develop in conjunction with coordinated behaviour of the ciliary band (Burke, 1983a,b). Although catecholamines are indicated, physiological proof is lacking that catecholamines are indeed involved with the nervous innervation of cilia in echinoderm larvae.

The larvae of polychaetous annelids provide an example at the extremely simple end of the invertebrate range and yet, even among these larvae there exists a diversity of behavioural and nervous organization. Because of the relative simplicity of the systems involved, correlations between behavioural activity and the neural transmission involved in its control may be more easily established than in more complex systems. These organisms are however, very small, 60 to 200 m across and study of the morphology involved must be conducted at the ultrastrucural level. Nervous differentiation and behaviour differ widely between planktotrophic (Marsden and Anderson, 1981; Marsden, 1982) and non-planktotrophic (Marsden and Lacalli, 1978; Bhup and Marsden, 1982) polychaete larvae. In non-planktotrophic larvae such as those of Arenicola cristata, Nereis virens and Capitella capitata the first nervous tissue to appear is laid down in the adult pattern. The larval nervous connections seen are restricted to segmental musculature (Marsden and Lacalli, 1978; Bell 1980; Lacalli, 1981a) and the paired eyes (Bhup an Marsden, 1982), structures that persist in the adult. On the other hand, in the planktotrophic trochophore larvae of the serpulids Spirobranchus polycerus (Lacalli, 1982), Galeolaria caespitosa (Marsden and Anderson, 1981; Marsden, 1982) and Spirobranchus giganteus (Marsden,

in preparation) there is a larval nervous system quite different in design from the initial components of the adult system which appears somewhat later. The pharmacology of the larval system is to date unknown. Harmothöe imbricata is intermediate in the planktotrophic, non-plantotrophic range since it is released as a planktotrophic larva only after 14 days of development (Holborow and Laverack, 1972). It was also the first polychaete larva to be studied with the electron microscope (Holborow, et al., 1969; Holborow, 1971; Holborow and Laverack, 1972). Holborow and Laverack (1972) found some evidence of neural structures but poor fixation prevented any extensive analysis of nervous organization or connection with effectors such as cilia or muscle. The following study examines at the level of the electron microscope, the morphology of larvae of H. imbricata with special emphasis on the development of neural structures. Since earlier work suffered from erratic and/or inadequate fixation, a basic requirement for the present study was the proper fixation of larval tissue. Considerable time was, therefore, devoted in this study to the development of a reliable method.

The finding of dense core granules in the larval nervous tissue, suggested the use of histochemical tests for aminergic neurons. Two techniques were employed: staining with neutral red and fluorescence following exposure to glyoxylic acid. Neutral red is a vital dye accumulated selectively by aminergic neurons (Stuart, et al., 1974). A modification of the glyoxylic acid technique appropriate for marine organisms (Occurr and Berlind, 1983) was used. Glyoxylic acid converts catecholamines to a highly fluorescent derivative (Cooper,

Bloom and Roth, 1978) with characteristic fluorescent excitation and emission maxima that allow for spectral identification.

Since electrophysiological techniques would be difficult in these very small and active polychaete larvae, $^{3}$H-2-D-Deoxyglucose studies (Faraco-Cantin, et al., 1980) were undertaken to demonstrate the cells involved in the transmission of sensory stimulation. Taken up by active cells, 2-DG, is a form of glucose which cannot be metabolized, and therefore accumulates as an indicator of local glucose metabolism in the nervous system. It was hoped that this preliminary experiment would label the putatively identified neural tissue, particularly the cerebral ganglion, in the larvae of Harmothöe imbricata.

## Materials and Methods

### Collection and observation of larvae

Adult H. imbricata were dredged in Passamaquoddy Bay, New Brunswick, in late April and early May of 1983. Observations on living larvae and preparation of larvae for electron microscopy were carried out at the Huntsman Marine Laboratory, where the worms collected were found to have already spawned and consequently females were brooding fertilized eggs. Healthy brooding female worms were selected and placed in bowls of seawater kept at 10°C. The water was changed on every second day. Larval release, the response of larvae to the light of the microscope lamp and the undulating pattern of swimming were observed in these bowls using a dissecting microscope. More detailed observations on structure and behaviour were made with the aid of a compound microscope on individual larvae placed in very small drops of water on a slide.

### Electron Microscopy

The small size of the larvae necessitated the invention of a sievelike fixation chamber. The protruding, sealed end of the cap of an Epindorph capsule was cut off. A small piece of nylon mesh (with hole diameter of 80 µm) was then "capped" in. Next, the outermost rim of the capped end and the tip of the pointed end were both cut off, resulting in a cylinder that would allow liquid drainage while the larvae were being supported on the mesh. Larvae were pippetted into the chamber which was then transferred from solution to solution (blotting on filter paper ensured proper drainage of each solution). During the final change of the embedding mixture, the mesh covered

insert (cap with cut ends) was pushed out with a fine dissecting needle. The larvae-covered mesh was then lifted off its support and embedded.

Two fixation methods were developed which yielded satisfactory results. Because of the spring breeding season, testing of fixatives in the fall and winter months was carried out on adult tissue and a single, small population of larvae. Larval neural and endodermal tissue did not respond as well to the final fixatives used as did the larval epidermal and adult tissue. The nature of the tissue rather than its location within the larva is probably responsible for the quality of fixation since muscle was preserved consistently better than neural tissue (Fig. 39). The first method which was developed consisted of a primary fixative containing 2% glutaraldehyde with 0.1 M cacodylate buffer and 4% paraformaldehyde in 0.28 M NaCl and a post-fixative containing 1 M cacodylate buffer and osmium in 0.28 M NaCl. The second fixative developed consisted of 2.5% glutaraldegyde with 0.4 M NaCl, at pH 7.4, in 0.2 M cacodylate buffer followed by 1% osmium tetroxide in 2.5% sodium bicarbonate. Both types of fixation were followed by alcohol dehydration and embedding in Spurr. In total, 12 batches of larvae (6 groups x 2 fixation methods) were fixed every 24 hours during 12 consecutive days.

Electron microscopy was confined to the larval episphere with special concentration on the anterodorsal ectoderm and the prototrochal region. Three each of 6 and 7 day old larvae and five each of 8 and 10 day old larvae were processed for electron microscopy. Silver sections were cut with a diamond knife on a

Sorvall MT-2 or MT-2B ultramicrotome and picked up on carbon-coated, formvar-covered, single slot, copper grids. The staining procedure consisted of hot (60°C), 1% uranyl acetate for 2½ hours followed by simplified lead citrate for 15 minutes. Sections were viewed with a Phillips EM 410.

Light Microscopy

At least five larvae of each of 6, 7, 8 and 10 days were sectioned at 1 to 2 um in thickness. These were dry sections cut with glass knives on the ultramicrotomes mentioned above. Sections were floated onto water droplets, spread with xylene fumes, and then dried for 2 minutes at 60°C. The alternation of thick, toluidine blue (0.1%) stained sections with thin sections was found to be a useful method for orientiation of material viewed in the electron microscope.

Staining for Catecholamines

A shipment of living H. imbricata was obtained from Marine Research Associates (St. Andrews, New Brunswick) in March, 1984. All had spawned before arrival resulting in a mixed population of larvae of various ages used in the following experiments.

Vital staining for aminergic cells:

Neutral red is a vital dye accumulated selectively in aminergic neurons (Stuart, et al., 1974). Larvae were incubated for 15 minutes in a 1% solution of neutral red in seawater at room temperature. Whole mounts were prepared with glycerine jelly. The three dimensional quality of the location of the stained cells in 10 day old larvae was retained by making a series of "optical section" photomicrographs which were then compiled into one schematic diagram.

Histofluorescence of Catecholamines:

The glyoxylic acid method of Occurr and Berlind (1983) requires an one hour incubation at room temperature in a solution containing 3% glyoxylic acid in seawater, 600 mM sucrose, and 20 mM Hepes buffer, buffered to pH 7.5 with NaOH. The control solution excluded the glyoxylic acid only. Incubated larvae were placed on a slide, air dried for one hour, heated for 2 minutes at 80°C and then photographed under oil immersion with a Leitz fluorescence microscope (200W high-pressure mercury lamp). Following incubation, glyoxylic acid and control preparations were handled in the same way.

Response to Light

Responses to light were tested in a silicone chamber built onto a glass plate 76 x 48 mm. The chamber, 76 mm long, 10 mm wide and 6 mm deep was opaque on the sides and at one end while the other end, made of clear plexiglass with plane parallel surfaces, provided a window through which light from the experimental lamp entered the chamber. Larvae were viewed and photographed from above with a Nikon camera. Tests were run on 2 ml aliquots of culture fluid with each aliquot containing an estimated 450 to 650 larvae. Tests were carried out in a darkroom where the only light was the experimental lamp and the camera flash placed below the glass-bottomed chamber. The testing procedure consisted of dark adaptation for 30 minutes followed by photographs taken at 3, 6, 8 and 10 minutes after the light source was turned on. In each trial, larvae were counted at 3 positions in the observation chamber; position A was a corridor at 0.22 - 0.45 cm from the window, position B at 2.45 - 2.68 cm and position C at 4.65 - 4.91

cm. A new batch of larvae was used for each colour of light tested (white, red, green and blue). Those larvae used in the white light trial were dark adapted for 30 minutes, photographed and then dark adapted again for 3 minutes and photographed to check the effects of the camera flash.

Experimental illumination was provided by an American Optical Microscope Lamp, at 6 volts, placed so that the ground glass filter at the end of the lamp housing was at the level of and 6 cm away from the window at the end of the observation chamber. For experiments using different wavelengths of light, a filter was placed directly in front of the lens of the lamp. The filters used were Kodak Wratten numbers 25 (red) which blocks transmission of all visible wavelengths except those greater than 550 nm, number 58 (Green) transmitting between 470 and 620 nm and number 47B (blue) transmitting between 360 and 510 nm. An adjusted Chi-square test (Adler and Foessler, 1968) was used to assess the asymmetry of the distribution of larvae at position A, B and C.

<u>$^{3}$H-2-D-Deoxyglucose Autoradiography</u>

The method used in this study was developed from that of Faraco-Cantin, et al., (1980). Larvae were incubated in 1.3 µCi of $^{3}$H-2-DG per ml. of filtered seawater for 24 hours at 10°C. Following incubation the larvae were washed with a few quick changes of fresh seawater to remove the radioactive seawater. The larvae (in the previously described fixation chambers) were immersed in Freon 23 cooled to the temperature of liquid $N_2$. The larvae were then quickly transferred into a precooled (-70°C) freeze-substitution solution of

5% $AlO_3$ in anhydrous acetone, re-stored at -70°C for 2 days and then allowed to reach room temperature slowly by first transferring the vial to -4°C for several hours. The larvae were then placed into a 50% maraglas and 50% anhydrous acetone solution for two hours (the solution was left uncovered to allow acetone evaporation), transferred to 100% maraglas and left overnight. A final fresh change of 100% maraglas the following morning preceded embedding in flatblocks. Thick (1 - 2 µm) sections were cut dry, mounted on glycerine drops in slides, dipped in Kodak NTB-2 nuclear emulsion and developed after 3 to 6 weeks.

# RESULTS

## General Development

In the developing Harmothöe imbricata larva, structural change proceeds along with an increasing capacity for behavioural response (Figure 1 and Table 1).

Female H. imbricata in bowls in the laboratory released larvae at a variety of stages corresponding to day 7 of development or later. Larval release can be induced by removing or damaging the adult elytra and it seems likely that the trauma experienced by females during collection and subsequent handling in the laboratory was in many cases sufficient to initiate early release. Larvae retained under the elytra are held by a mucous-like material. Observations on living larvae at the Huntsman Marine Laboratory suggest that they develop in the same fashion regardless of the time of release from the parent. All larvae began to swim when the prototrochal cilia developed (larvae still attached by mucous strands, when freed, could swim away) and began to respond positively to light when the eyes appeared. Larvae swim in a straight line while rotating about the long axis of the body.

At 6 to 7 days of development, the larvae possess an apical tuft, the first obviously differentiated structure to appear. The gut cells form a solid mass and stain more darkly with toluidine blue than do surrounding ectodermal cells. Within the small blastocoel, islands of mesenchymal cells are present. By day 7 a prototrochal ring of cilia protrudes outward at the equatorial region and a few nerve processes and associated vesicles appear in the blastocoel at the same level of

Figure 1. (a) Drawing of a six day old larva as seen from the side. Apical tuft (at), cerebral mass (cm), prototroch (pr), stomodaeal depression (s), endodermal gut mass (g).

(b) Drawing of a 10 day old larva as seen from the side. Apical tuft (at), cerebral ganglion (cg), eye (e), prototroch (pr), stomodaeum (s), gut lument (gl).

Fig. 1

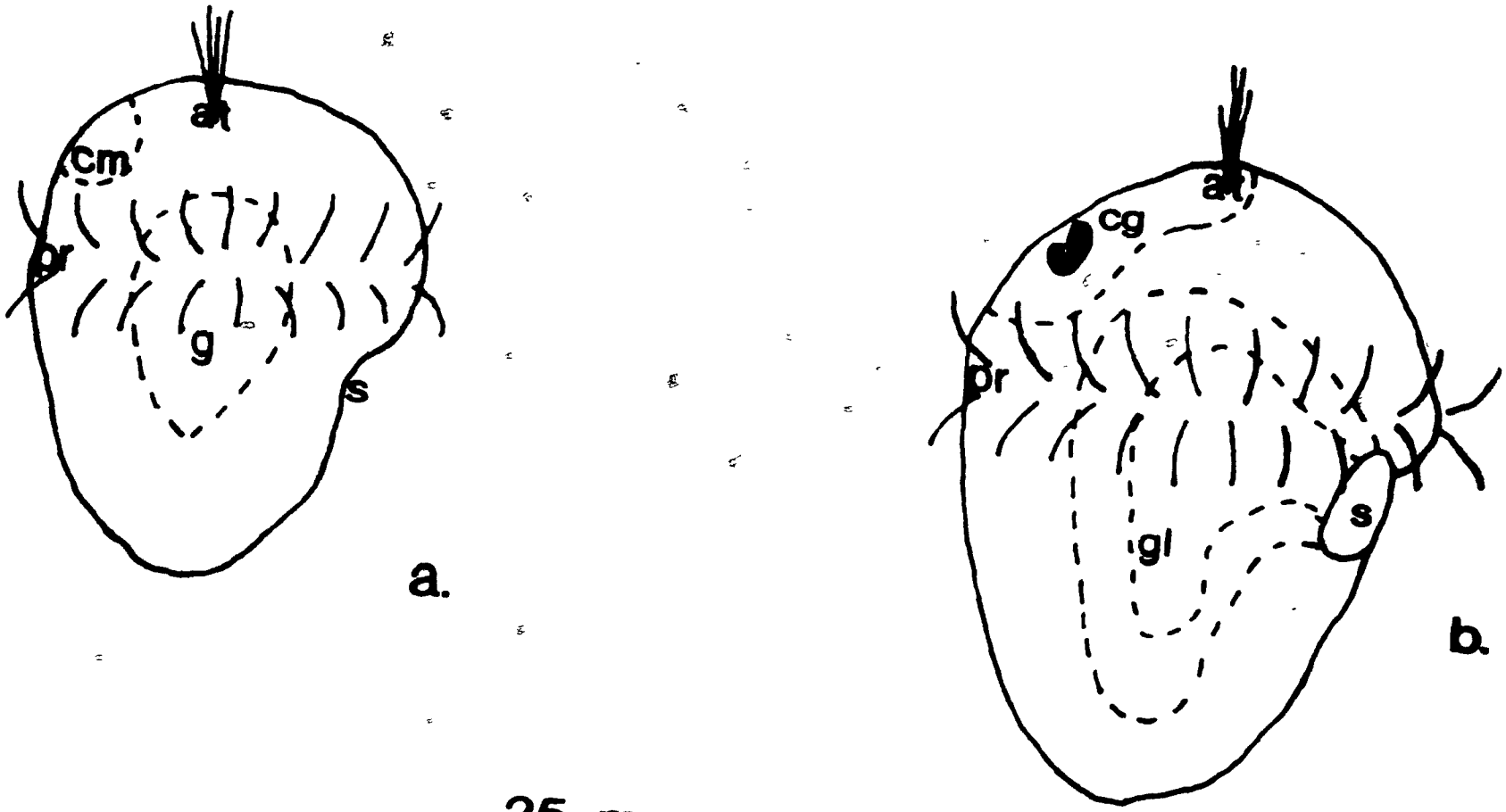


Table 1.

| Structure or Behaviour | Day 6 | Day 7 | Day 8 | Day 10 |
|---|---|---|---|---|
| Apical organ | x | x | x | x |
| Prototrochal cilia | - | x | x | x |
| Cerebral masses (paired) | - | x | x | - |
| Eyes | - | some | x | x |
| Stomodaeum | - | x | x | cnx. made with gut |
| Processes & vesicles | - | x | x | x |
| Neuropil | - | - | x | x |
| Cerebral ganglion | - | - | - | x |
| Muscle | - | - | x | x |
| Swimming | - | - | x | x |
| Positive response to light | - | - | x | x |
| Evoked and spontaneous responses | - | - | on day 9 | x |

the body. At the same time two patches of dividing cells, the rudiments of the cerebral ganglion, appear in the anterior ectoderm, and the stomodaeum begins to form as a depression on the ventral surface of the body. The stomodaeum continues to invaginate and proliferate until it eventually meets the gut lumen by day 10. At 7 days, larvae are normally still found in clusters, attached to each other in a spoked configuration by means of mucous strands. Such clusters of larvae, when free of the parent can be seen to revolve in a wheel-like fashion: the propelling force for this rotation orginates from individual larvae, each spinning about its longitudinal axis from right to left (clockwise as seen from above).

On the 8th day of development, a rhabdomeric ocellus appears within each cerebral mass; a medial cerebral neuropil developes and there is an increase in the number of nerve processes in the prototrochal nerve. Patches of muscle are now present in the blastocoel. After the appearance of the eyes, individual larvae, separated from the group under the parental elytra, exhibit a positive response to light. An analysis of this response in 8 to 10 day old larvae is described in the section on Response to Light.

By day 10, the anlage of the cerebral ganglion has become a distinctly bilobed structure, consisting of 2 lateral cell masses connected by a large neuropil. At this stage the hyposphere has begun to increase in length and the larva are about 250 μm long. Three types of neural processes containing three different populations of vesicles/granules can be seen at this stage and are described in the section on the Prototrochal Nerve. Synapse-like structures consisting

of clusters of small, lucent vesicles and some membrane specializations occur between nerve processes and the basallar folds of prototrochal cells.

The larval prototroch is a double ring of at least 8 cells per ring all with large nuclei with very large, darkly staining nucleoli. There are mesenchymal cells in the blastocoel under the prototroch; they are especially numerous ventrally, on either side of the stomodaeum. Although musculature is first seen on day 8, muscular contractions in response to slight mechanical stimulation take place only on the ninth day of development: when touched at the prototroch, ciliary beating was seen to slow down, followed by twitch-like contractions in the mid-region of the body. These responses quickened and became more obvious by day 10. At this time, larvae also begin to respond to stimulation by a longitudinal contraction of the body alternating with the mid-regional circular contractions.

## Ultrastructure

### Prototrochal Nerve

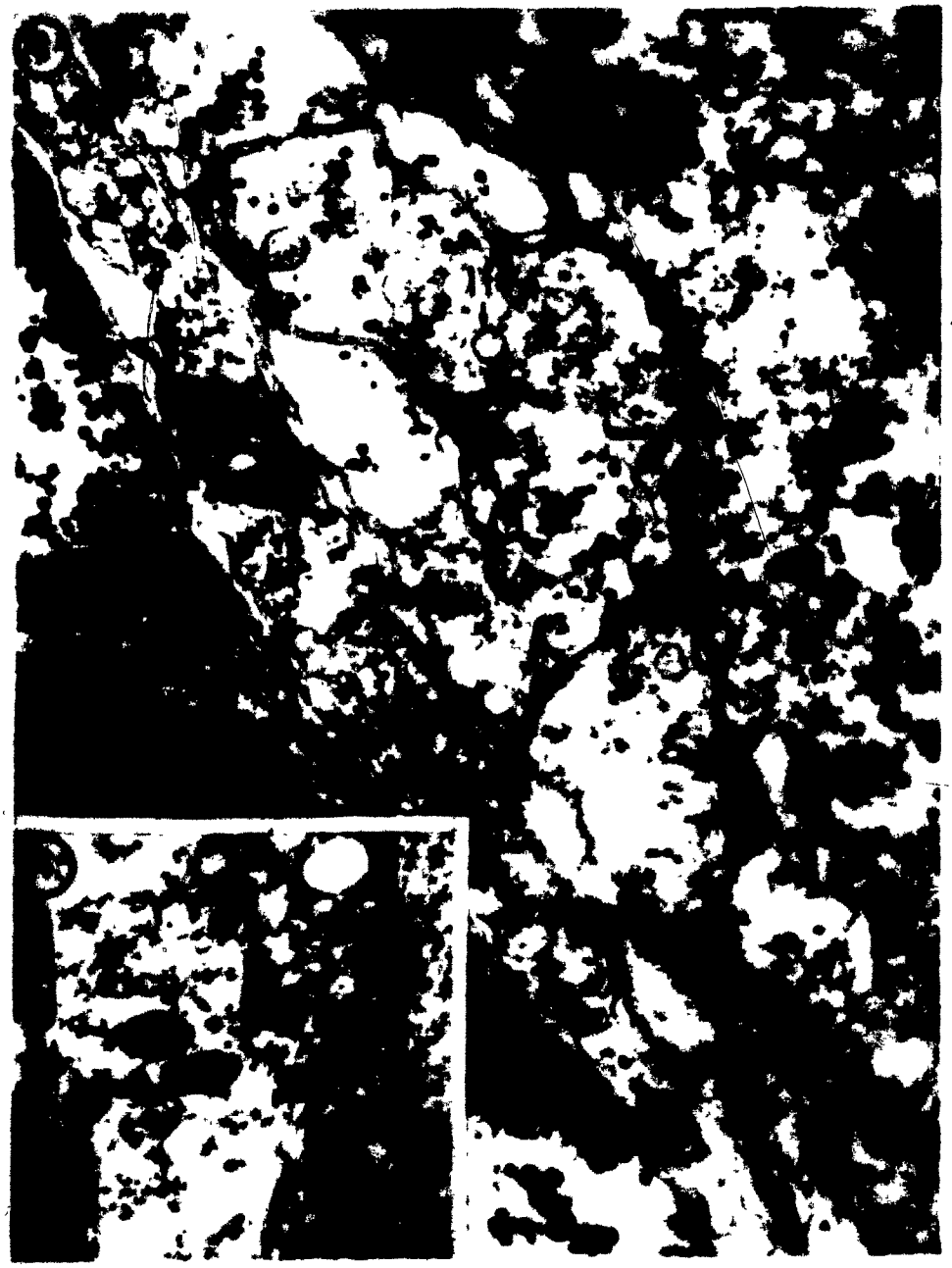
Both prototrochal and cerebral cells can be first seen at the light microscope level between day 6 and 7 of development (Fig. 2). Within the next 24 hours, a small nerve ring appears on the blastocoelic surface of the prototroch (Fig. 3). This early nerve consists of approximately five processes carrying large lucent vesicles (65 to 120 nm) and a few scattered dense-core granules (60 to 100 nm) illustrated in Fig. 4. No signs of synaptic specialization were found at this stage. There is no indication that apical cells give rise to neurite-like processes.

Figure 2. Section of 7 day old larva with apical organ (ao), prototroch (pr), dividing cerebral cells (cm), and the beginning of the stomodaeum (s). X 2112.5.

(2)

Figure 3. An early nerve containing 5 processes in the prototrochal region in a 7 day larva. Processes carry large, electron-lucent vesicles (llv), 65 to 120 nm in diameter. X 53950.

Figure 4. (Insert) An early (7 days) nerve process containing a few dense cored granules (dcg), 60 to 100 nm in diameter. No synaptic specializations were found at this stage. X 17500.



The apical organ persists through all the larval stages studied here (i.e., up to day 10 of development). In Harmothöe imbricata, the simple, 4-celled apical organ consists of one ciliated cell ($A_1$) with a tuft (approximately 20 µm long by 10 µm wide) of asymmetrically arranged cilia (Holborow, 1971), and three non-ciliated cells (Fig. 5). Two of the latter ($A_2$, $A_3$) are smaller than, but have cytoplasm similar in composition to that of the ciliated cell; in the fourth cell ($A_4$), both nucleus and cytoplasm are less dense. All four cells contribute to the apical surface of the larva and contain granules characteristic of the ciliated cells of both the apical organ and the prototroch. These granules (C-granules) are 1 µm long by 0.4 µm wide on average, almond shaped and lie close to the cell surface. The cytoplasm of ciliated cells of prototroch and apical organ can each be roughly divided into three layers; a superficial layer containing ciliary rootlets and C-granules, a dense mid-level population of mitochondria, and a basal layer containing numerous Golgi, ribosomes and the nuclei. As mentioned, neurites have not been seen to emanate from the apical cells although the cerebral commissure, derived from cells in the cerebral masses, consistently develops directly beneath the apical organ.

On day 8, after further cerebral cell proliferation and the appearance of an eye in each cerebral mass, the number of processes in the prototrochal nerve has increased and small, electron-lucent vesicles (40-60 nm) are apparent (Fig. 6 and 7). The latter are often found in clusters in which an electron-dense interstitial matrix is involved. The prototrochal nerve processes are usually found adjacent

Figure 5. 4 cells ($A_1$, $A_2$, $A_3$, $A_4$) of the apical organ of the Harmothöe imbricata larva. Only one ($A_1$) is ciliated although all 4 contribute to the apical surface. C-granules (C) and mitochondria (M).

Figure 6. Synapse-like clusters (arrows) in electron-lucent prototrochal nerve processes intermingling with basallar folds of prototrochal cells. X 32500.

Figure 7. (Insert) An enlargement of a synapse-like cluster of small, electron-lucent vesicles that are each 40 to 60 nm in diameter and an electron-dense interstitial matrix. X 95550.

to and intermingling with basal folds of prototrochal cells. Characteristically, the prototrochal basallar folds contain snuggly fitting mitochondria and lack vesicles and granules. These folds may be associated with muscle as well as nerve processes (Fig. 8). Muscle is very sparse at this stage and its orientation is difficult to judge. Observations at both electron and light microscope levels suggest that in the 10 day larva there is a group of muscle cells ensheathing the proximal end of the stomadaeum, near its coalescence with the endodermal gut (Fig 9) Two muscle insertion sites have been seen, one at the point where the invaginating stomodaeum meets the endodermal cells (Fig. 10) and a second on the ciliated, stomodaeal epithelium (Fig. 11). Muscle fibers are found passing through the blastocoel of the episphere as early as 8 days and are accompanied by nerve processes by day 9 or 10. These processes appear to arise as a branch of the prototrochal nerve (Fig. 12). Electron-lucent vesicles in the processes have been observed to form omega profiles on the neurite plasma membrane adjacent to the muscle membrane (Fig. 13). Contractile filaments with scattered mitochondria make up most of the muscle cell cytoplasm while ribosomes and other organelles occur in electron lucent areas.

As the complexity of the prototrochal nerve increases (Fig. 14) presynaptic clusters as well as pre- and post-synaptic membrane specializations suggest functionality at a time when behavioural observations indicate the onset of larval response to mechanical stimulation (Fig. 15). There appear to be 3 populations of vesicles/granules in the 10 day larval nervous system. One population consists

Figure 8. Basallar folds of prototrochal cells (prc), intermingling with electron-lucent processes (lp) of the prototrochal nerve. Mitochondria (mi). X 26000.

Figure 9. A group of muscle (m) cells at the point where the stomodaeum (s) meets the endodermal gut (g). X 640.

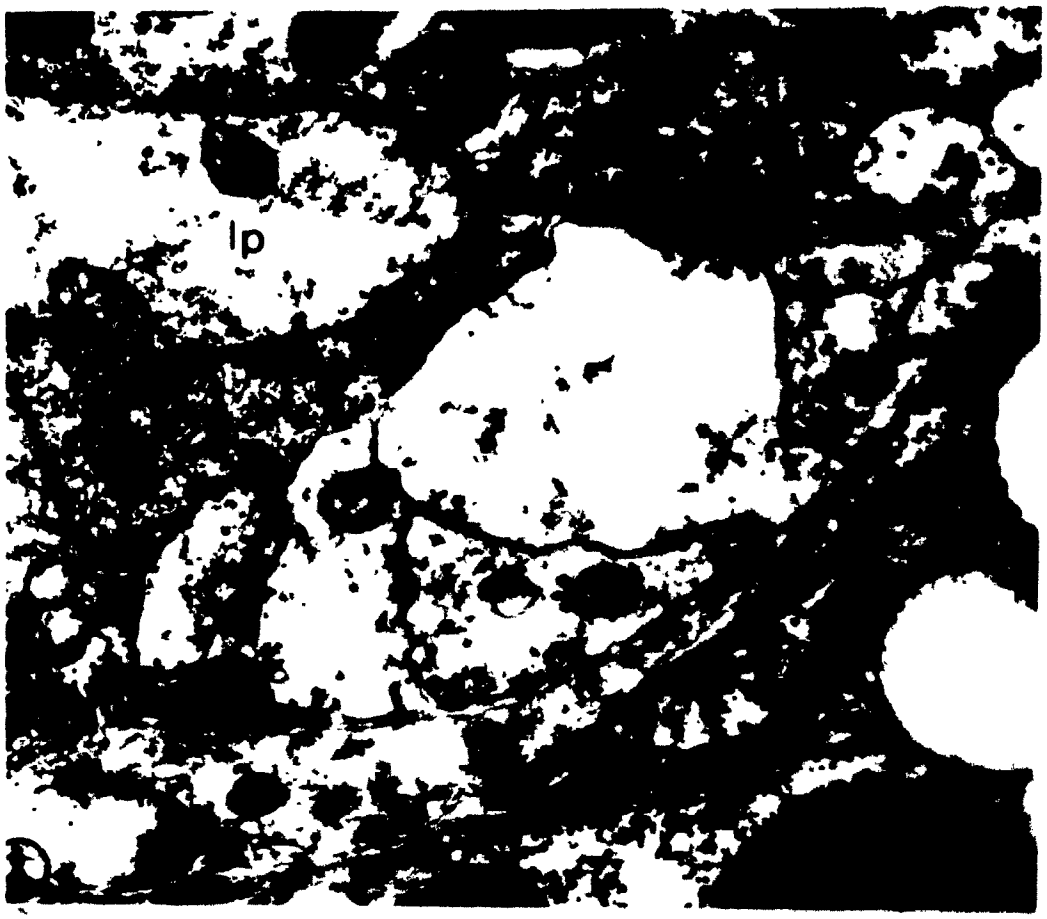



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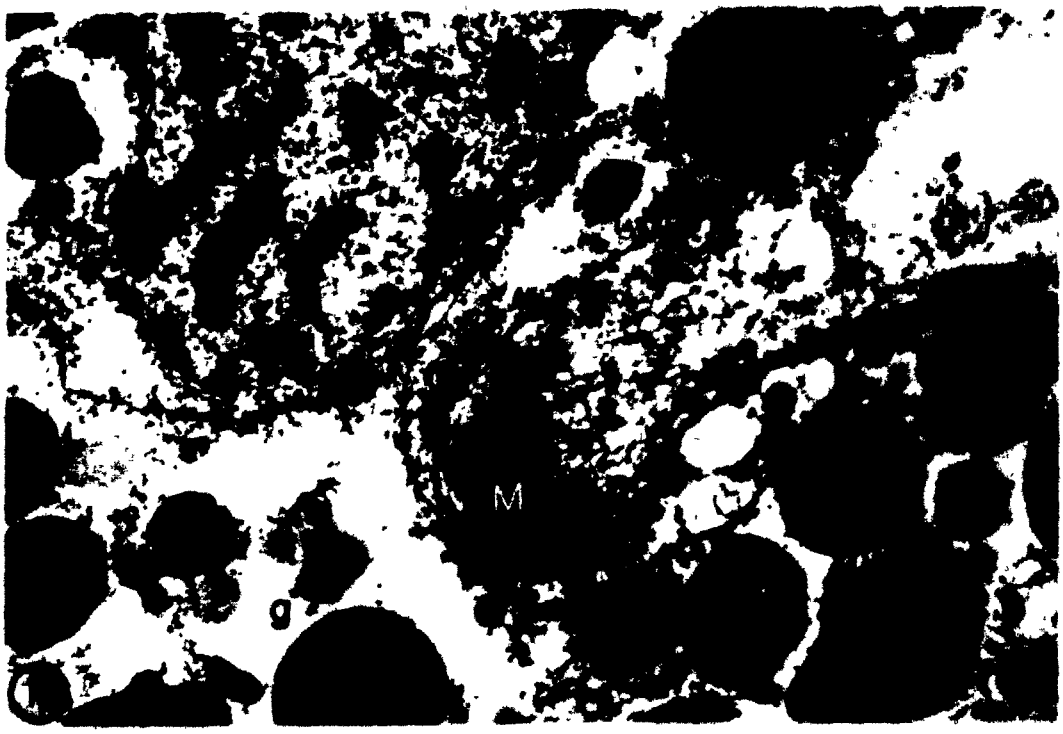
Figure 10. A muscle insertion point on the anterior face (M) of the gut mass (g) in the region where the stomodaeum contacts the endoderm. X 14690

Figure 11. A second muscle (M) insertion point on the stomodaeal epithelium (s). Cilia (c). X 14690.


M
g

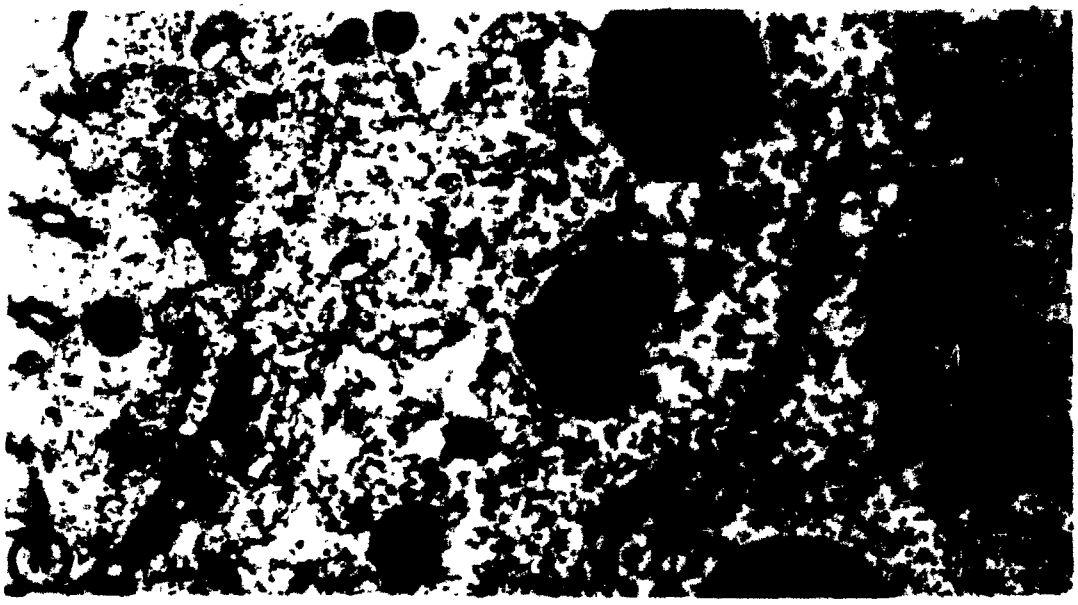


Figure 12. Section of a 10 day old larva. Electron-lucent processes (lp) serving both prototrochal ciliated cells (prc) and stomodaeal muscle (m). Neuropil (np), cerebral ganglion (cg), stomodaeum (s).

Figure 13. Electron-Lucent vesicles forming omega profiles (small arrows) on prototrochal nerve processes (1p). Mitochondria (M). A synapse-like cluster (large arrow) lies adjacent to specializations of the neurite. X 42900.

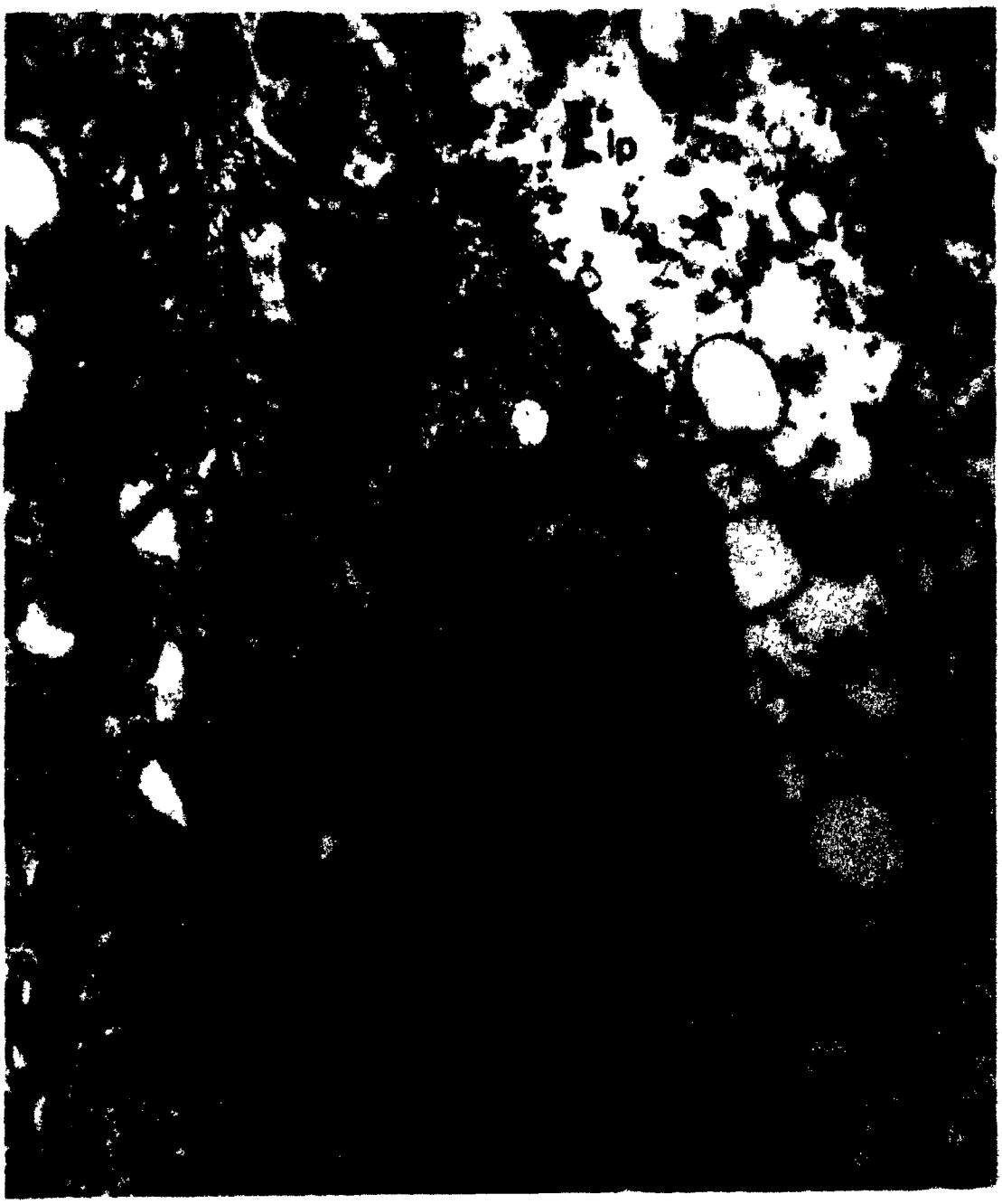

lp

Figure 14. An oblique section through the larval mid-region revealing a gland cell (gc) in the anterior ectoderm and the prototrochal nerve processes (lp) following and intermingling with basallar folds of the prototrochal cells (prc). Mitochondria (M). X 11200.

N

Figure 15. Prototrochal nerve processes containing synapse-like clusters presynaptic (arrows) to prototrochal cells. X 26000.

Figure 16. Electron-dense processes (dp) are unique and confined to the neuropil. They contain a high density of dense cored granules (arrows). X 5785.

of dense-core granules (60 to 100 nm). These granules are scarce in prototrochal nerve processes before the appearance of the eye but become more abundant as cerebral masses proliferate and neuropil formation begins. At 10 days dense-core granules are characteristically found in processes of various electron densities in the neuropil (Fig. 16) and are common in the later appearing processes (containing prototrochal cell-like cytoplasm) which interdigitate with muscle in the prototrochal region (Fig. 17). The granules are never found in clusters, and are only rarely associated with vesicle clusters. A second population consists of small (40-60 nm), electron-lucent vesicles, first seen in 8 day larvae. They are distributed singly throughout axons and are also found in clusters together with a dense, interstitial matrix located, in 10 day larvae, at (Fig. 18a) or near (Fig. 18b) membrane specializations which are uniform in thickness and smoothly contoured. At these synapse-like sites, the cleft (20 nm wide) between the two opposing membranes characteristically contains electron-dense material. Clusters of vesicles appear only on one side of the cleft. As mentioned previously, these small, electron-lucent vesicles have also been seen to form omega profiles on the neural plasma membrane at the surface of the muscle (Fig. 13). The third population of vesicles are large (65-120 nm), electron-lucent and are first seen in the prototrochal nerve as early as 6 days (Fig. 18b). They do not themselves form clusters although they are sometimes associated with the periphery of the clusters of small, electron-lucent vesicles (Fig. 19). Isolated coated vesicles are occasionally found in prototrochal nerve processes (Fig. 19).

Figure 17. A third type of nerve process (pp) containing cytoplasm very similar to prototrochal cell cytoplasm (prc), but unlike the latter, contains the usual three populations of vesicles/granules, although no synapse-like structures are seen. This type of fiber interdigitates with prototrochal basallar folds (prc) and with muscle (m). Gut (g).
X 18200.

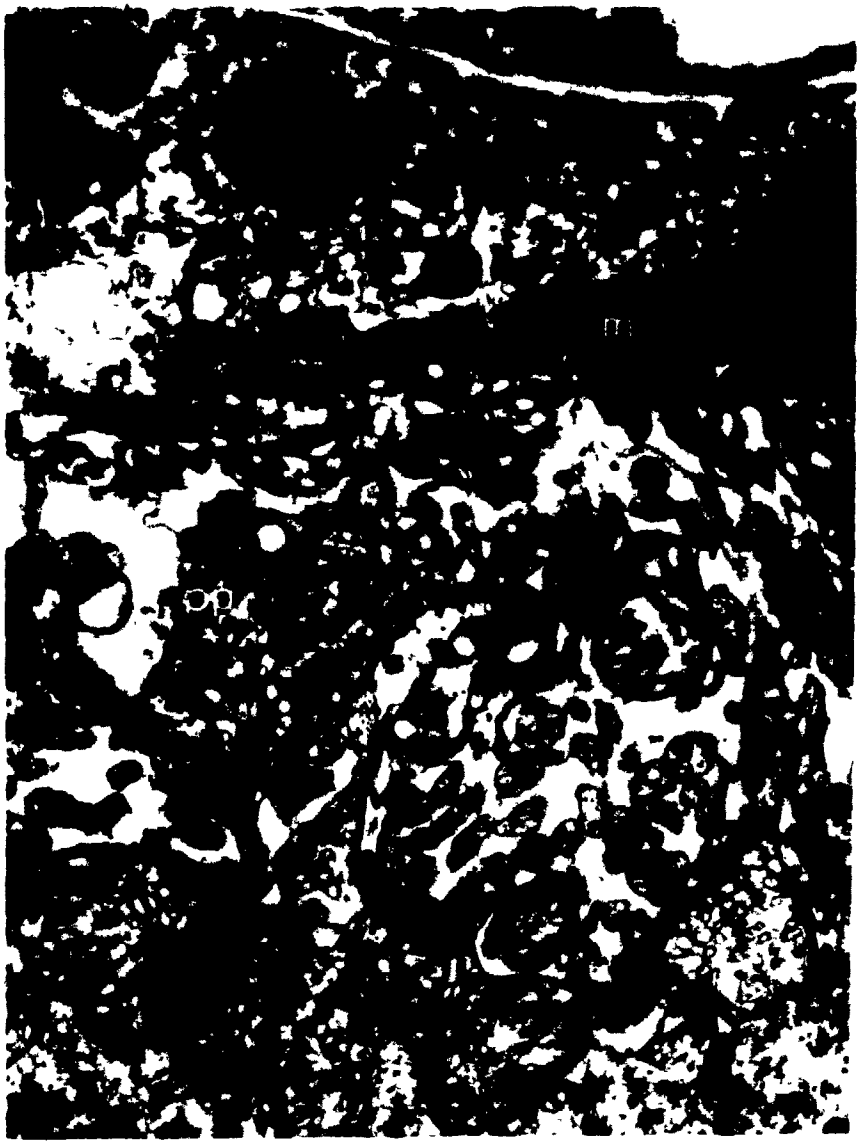


Figure 18. Synapse-like structures consisting of clusters of vesicles situated at (18a) or near (18b) membrane specializations which are uniform in thickness and smoothly contoured. X 71500.

Fig. 18

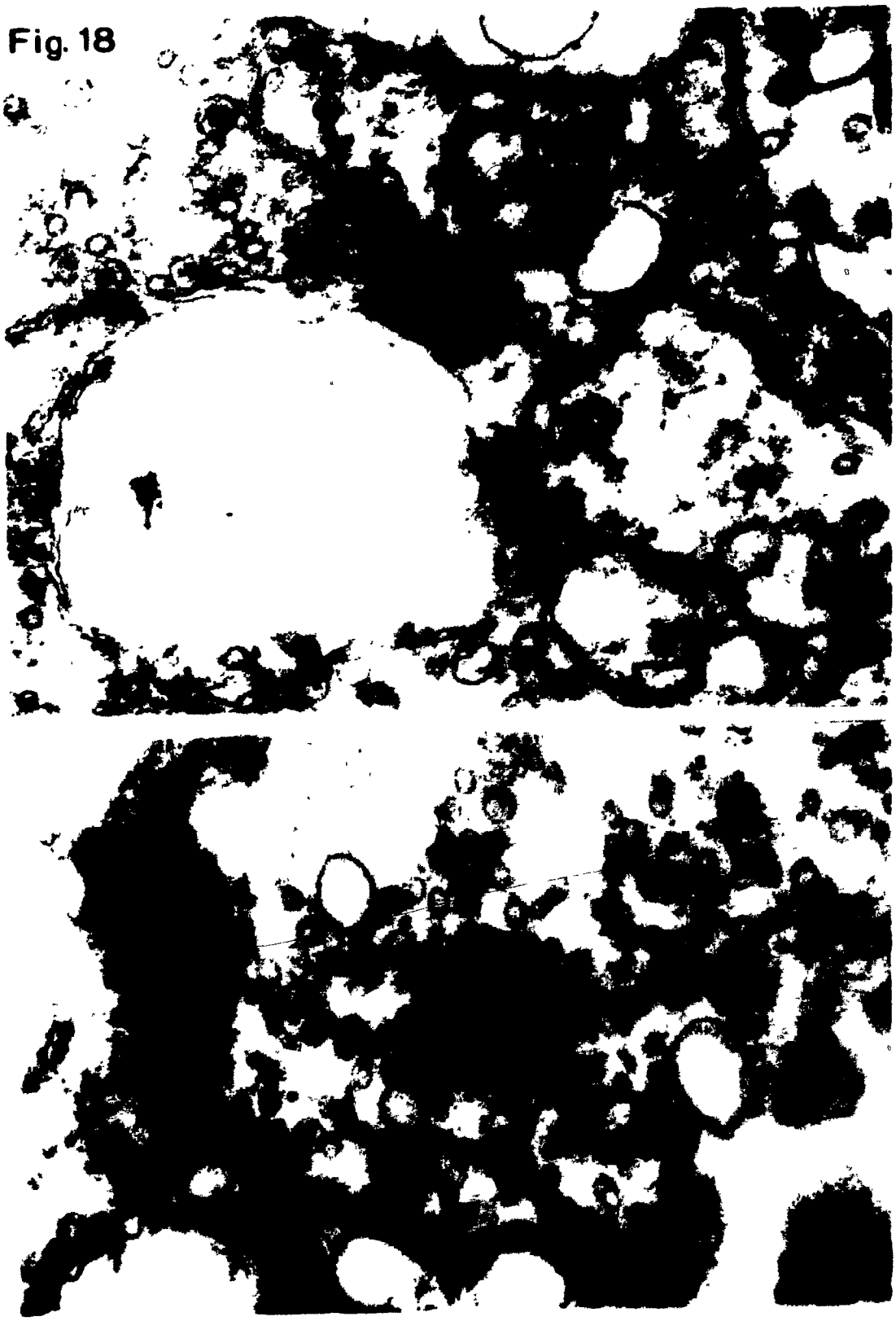
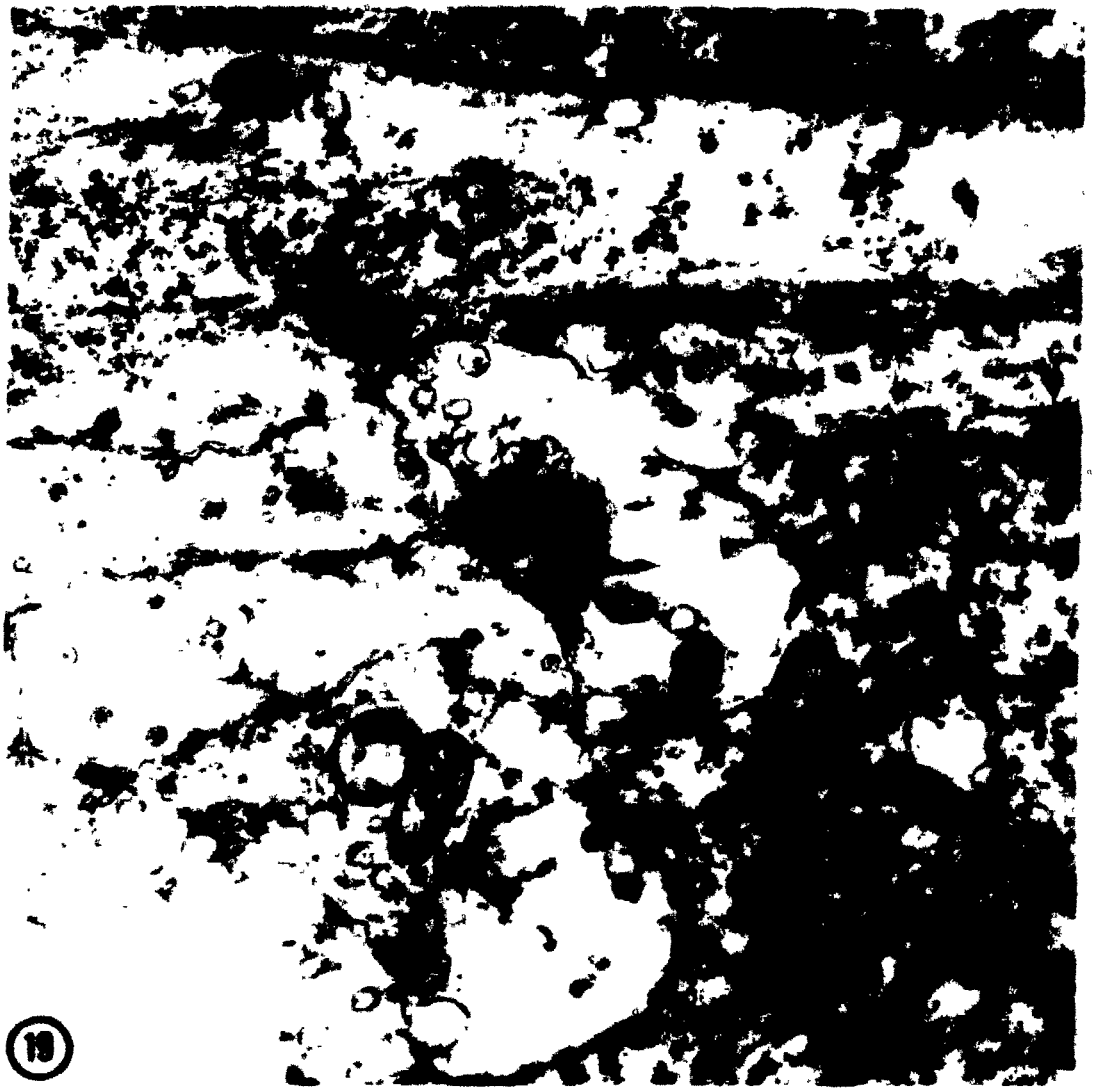


Figure 19. Large, electron-lucent vesicles (small arrow) are sometimes associated with the periphery of synapse-like clusters and coated vesicles are occasionally seen in nerve processes. X 42900.


10

At 10 days of development, muscle is associated with a third type of process which is ultrastructurally distinct from earlier prototrochal nerve processes, resembling rather, in cytoplasmic structure, the basal folds of the prototrochal cells. These processes contain vesicles/granules similar to those found in other neurites. No sign of synaptic specializations have been observed in the later-appearing processes. A coated vesicle in such a process is shown adjacent to the muscle fibre membrane (Fig. 17).

Cerebral Ganglion and Eyespots

In the 7 day larva the posterior border of the paired cerebral masses lies about 10 μm anterior to the prototroch. At the anterior and posterior border of each mass is a slender gland cell, each spanning the thickness of the anterior ectoderm. Figure 20 demonstrates the posterior gland cell and, approximately 15 μm above it, the eyespot in the 8 day larva. The two eyes are bilaterally symmetrical in position and are characteristically rhabdomeric, lacking ciliary structures of any kind. Each eye consists of two cells. One cell forming the pigment cup into which are inserted the microvillar endings of the second cell, the receptor cell. All other receptor cell components lie outside the eyecup. Holborow and Laverack (1972), using both scanning and transmission electron microscopy described a group of eye cilia originating from a cell superficial to the eye. However, in transverse sections similar to those used by these authors, no such ciliated cell was seen. A 2-3 μm layer of membrane bound electron-dense pigment granules lines the 4 μm deep eyecup (Fig. 21). The pigment cell nucleus lies immediately

Figure 20. A cerebral gland cell (gc) in the anterior ectoderm at the posterior margin of the cerebral ganglion (cg). Pigment granules (pg) of the eyecup can be seen above the pigment cell nucleus (pn). The large, light staining nucleus (rn) nearest to the blastocoel belongs to the receptor cell. X 5785.

Figure 21. Electron-dense granules (pg), which have dissolved in this case, in the pigment cell form a cup that contains the folded membranes (fm) of the receptor cell. The pigment cell nucleus (pn) lies immediately beneath the eyecup. Membrane-bound spaces (ms), mitochondria (M), larval surface (*). X 14690.

C

beneath the eyecup and is closely bounded by the cell membrane such that the surrounding layer of cytoplasm is at most 0.3 µm thick. The nucleus measures 6.5 µm at its broadest and tapers gradually to 3 µm over a length of 4 µm. It is noticeably more darkly staining than the receptor cell nucleus (Fig. 22). The eyecup faces outwards and is directed laterally and slightly anteriorly. The receptor cell spans the full thickness (25 µm) of the ectoderm. The outer end of the cell which projects into the hollow of the eyecup contain two layers of folded membrane as well as mitochondria, ribosomes and large, irregularly-shaped membrane bound spaces (Fig. 21). Immediately outside the pigment cell, only the receptor cell body flattens, curves medially, and gives rise to a neurite which following a bundle of neurites arising from other cerebral cells. This bundle appears to travel posteriorly through the blastocoel towards the prototroch (Fig. 22).

Figure 23a,b and c represents serial sections through the layer of evaginating microvilli abutting against the side of the eyecup which is closest to the larval surface (Fig. 21). The microvilli number at least 325, contain an electron-dense core and measure about 0.1 µm in diameter (Fig. 23a). In longitudinal sections (Fig. 24), the layer of microvilli appears to be at least 1.0 µm thick.

The second, inner layer of membrane foldings results from invaginations into the cell body of the same membrane, occurring in between and parallel to the long axes of the microvilli. The two distinct layers of membrane foldings are therefore formed by alternating evaginations and invaginations of the same receptor cell

Figure 22. The receptor cell spanning the anterior ectoderm. Receptor cell microvelli (fm), receptor cell nucleus (rn) at a position below the pigment cell nucleus (pn). At the base of the receptor cell a neurite (*) travels posteroventrally to join a bundle of neurites arising from other cerebral ganglion cells. X 5785.

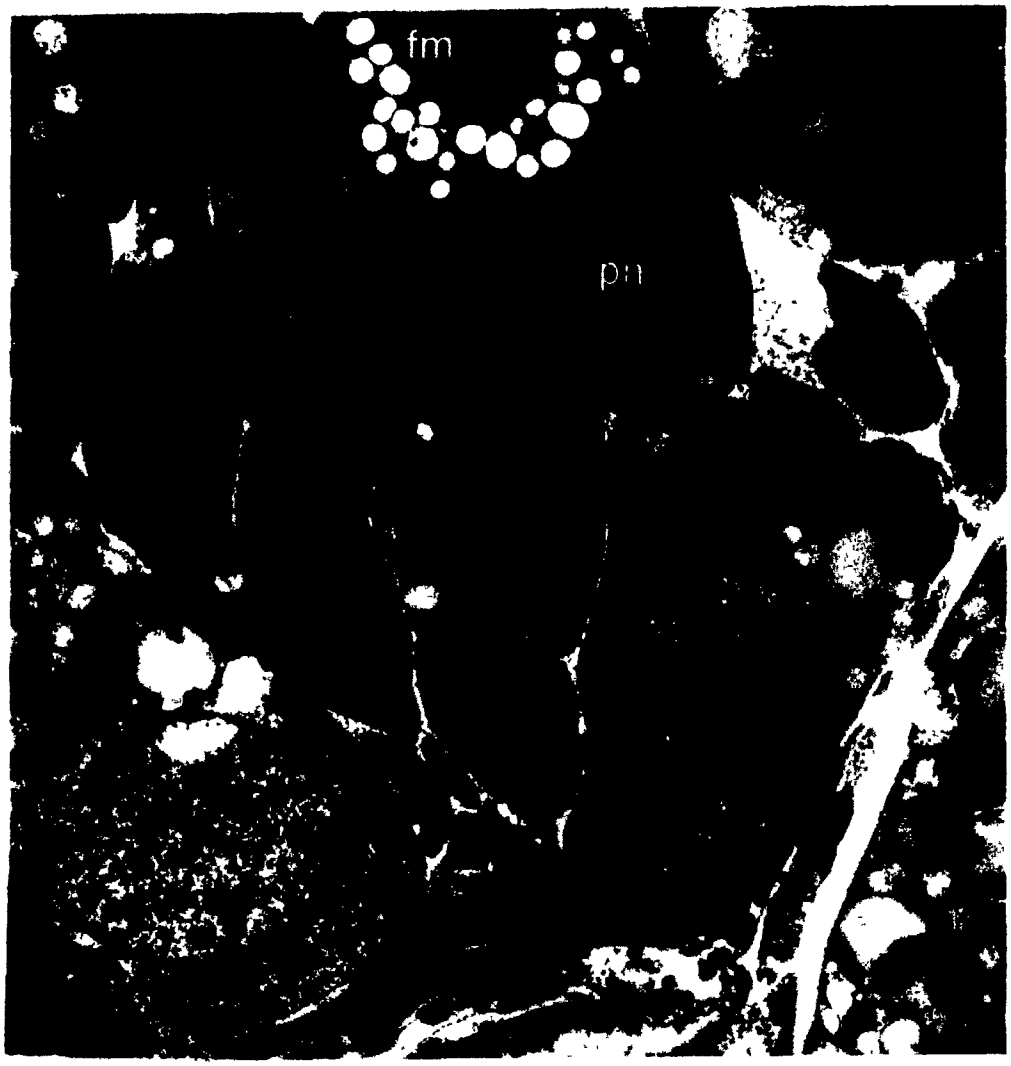

fm
pm

Figure 23. Serial cross sections through the first layer of evaginating microvilli, (a) furthest from inner surface of eyecup, (c) closest to inner surface of eyecup. (23a) X 24900 (23b,c) X 8400

Fig. 23

Figure 24. The inner membrane layer consists of invaginations of the same receptor membrane that evaginates to form the outer microvillus layer. The two layers are usually in a single plane. The invaginations are between 12 and 14 nm in diameter and extend approximately 1.5 μm into the receptor cell while the 1.0 m thick layer of evaginations consists of membrane foldings which are 0.1 μm in diameter

X 53950.

in diameter, are approximately 1.5 μm long and are associated with an electron-dense material which obscures their endings (Fig. 25) in the body of the cell. In all but one case, the two layers of folded membrane were found to lie in the same plane (the individual foldings being parallel to each other). However, in one instance (Fig. 26), the invaginated layer appears to be perpendicular to the evaginated layer and to be curved in a disorderly fashion.

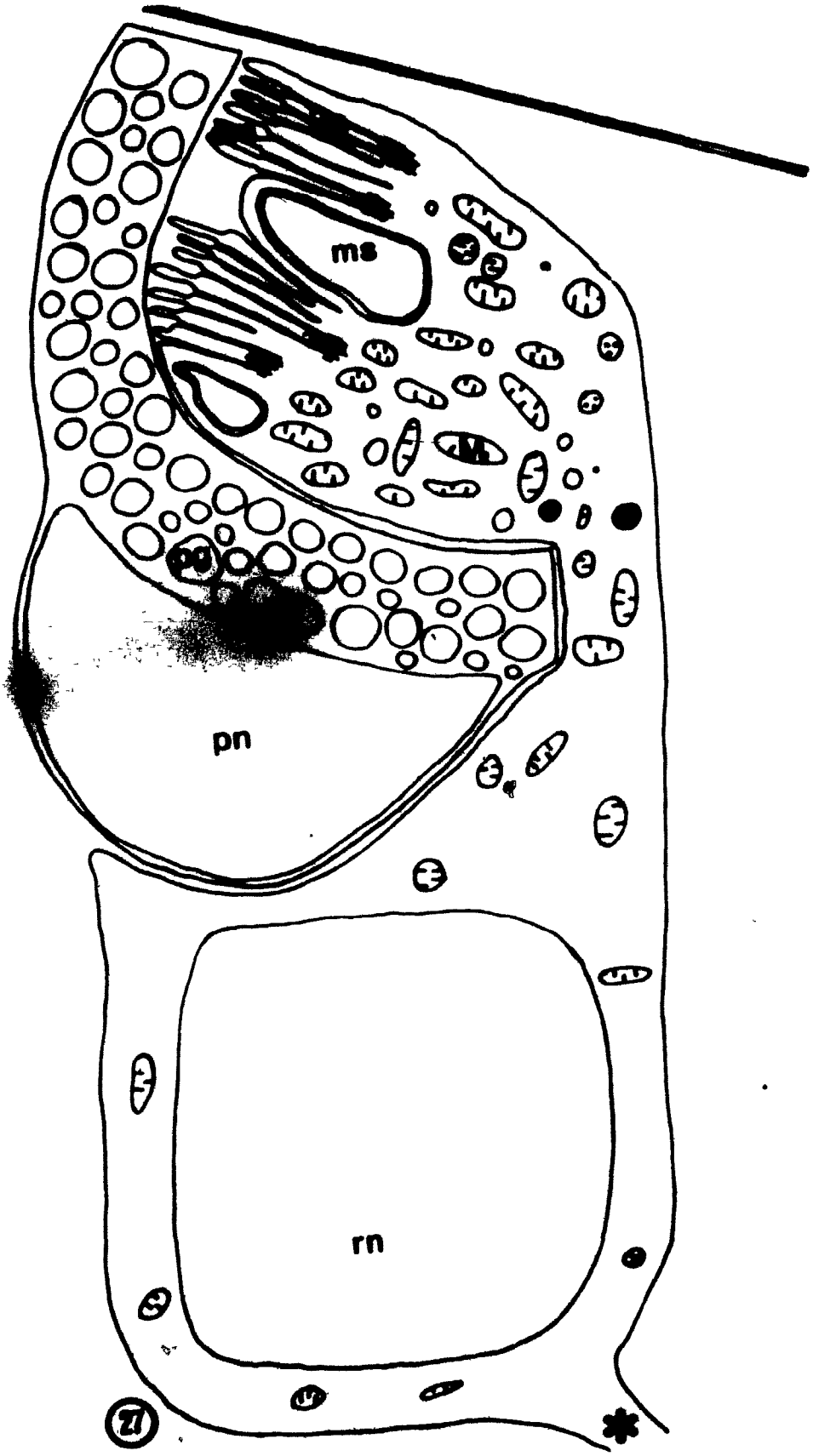
Holborow and Laverack (1972) have diagrammatically represented the larval eye of H. imbricata in a scheme which closely resembles that observed in the present study. There are, however differences between the two studies. A schematic drawing in the Holborow and Laverack (1972) account indicates no difference in the size of the membraneous projections of the two layers. In the present study a clear difference has been seen; the inner invaginations being much thinner and longer than the evaginated microvilli (Fig. 27). Also, the inner invaginations are associated with an electron-dense matrix, mentioned previously. In addition, the membrane-bound spaces in the vicinity of the invaginations found in this study are not described by Holborow and Laverack, and resemble the irregular membrane folds attributed to osmium fixation artifacts in Planarian photoreceptors (Röhlich, 1966). It is thought that these artifacts derived from the breakdown and reorganization of the membranes of preexisting microvilli, Eakin (1965) confirmed these results while studying the frog retina. Glutaraldehyde was found to have similar effects on the ciliary epithelium of rabbits (Tormey, 1963).

Figure 25. The invaginations are associated with an electron-dense matrix at their ends in the eyecup region of the receptor cell. X 71500.

Figure 26. In one case, the evaginated (em) and invaginated (im) layers of membrane appear to be perpendicular to each other. The inner, invaginations are also arranged in an unusually, curved and disordered fashion. Pigment granule (pg). X 32500.

C

Figure 27. Drawing of larval ocellus. Pigment granules (pg) form a cup to shield evaginated (em) and invaginated (im) layers of receptor cell membrane. Membrane-bound spaces (mb) and mitochondria (M) make up most of the remaining eyecup region of the receptor cell. Immediately below the pigment cup is the pigment cell nucleus (pn), the receptor cell nucleus (rn) lies below the pigment cell. A neurite (*) leaves the base of the receptor cell.


ms
pn
rn

Following the appearance of an eyespot in the center of each of the two cerebral masses, a neuropil (the rudiment of the cerebral commissure) begins to form beneath the apical organ. By day 10 of development, the paired cerebral masses together with the connecting neuropil have merged to form a bilobed cerebral ganglion occupying most of the dorsal-anterior ectoderm of the larval episphere (Fig. 28). Along with the usual electron-lucent processes, a second, very conspicuous type of electron-dense process can now be seen in the neuropil. Vesicles/granules are found in both types of processes with electron-lucent vesicles predominating in the electron-lucent processes and electron-dense cored granules in the electron-dense process (Fig. 16). No signs of synaptic specialization were seen in the neuropil at any stage observed in this study.

Autoradiographic study of larvae exposed to $^{3}$H-2-D-Deoxyglucose indicates an accumulation of the tracer in the apical region only (Fig. 29a,b). The labelling was in general light with a patch of somewhat greater density in the vicinity of one of the eyespots. In general the results of this technique were inconclusive and not helpful in understanding the morphology of the larval nervous system.

## Evidence for Catecholamines

### Neutral Red

Neutral red is a vital dye which accumulates selectively in aminergic cells (Stuart, et al., 1974). In treated larvae the cerebral masses, a double ring associated with the prototroch, a network of processes in the episphere and a few large cells or groups of cells in the posterior part of the body are stained very clearly

Figure 28. A 10 day old larva in which the endodermal gut (g) contains a lumen (gl) continuous with that of the stomodaeum (sl). Muscle (m) is seen in the region where the two cavities meet. A bilobed cerebral ganglion (cg) is connected by a neuropil (np) beneath the apical organ (ao). Neurites travel from the cerebral ganglion to the prototrochal ciliated cells (prc). Various gland cells (gc) are apparent in the larval ectoderm. X 1787.5.

28

Figure 29 Autoradiographic labelling in 9-10 day larvae exposed to 2-DG was found mostly in the larval episphere and particularly in the vicinity of one of the eyespots (arrows). Apical organ (ao), prototroch (pr). a transmitted light 560 x, b dark field 550 x

Fig. 29a.

(Fig. 30a,b,c). In these preparations there was no satisfactory evidence for a connection between the two prototrochal ring nerves (one anterior and one posterior to the prototroch) or for any contact between the eye and nerves of episphere. The prototrochal rings were irregular in shape, suggesting nerves of variable diameter

Glyoxylic Acid

Treatment with glyoxylic acid produced a prolonged blue-green fluorescence in a pattern which corresponded well with that resulting from staining with neutral red (Fig. 31a,b). This pattern of fluorescence was completely absent in control larvae (Fig. 31c). Autofluorescence, brilliant in the prototrochal cilia and less striking in the gut, was seen in all larvae.

The neutral red and glyoxylic acid results indicate a catecholaminergic component to the nervous system which corresponds, at least in part, with the distribution of neurites containing electron-dense cored granules as seen in the ultrastructural study. Figure 32 is a schematic diagram based on the above two techniques and on ultrastructural studies, tracing the distribution of catecholaminergic cells found in larvae of Harmothoe imbricata.

Response to Light -- A Preliminary Study

When exposed to a source of white light larval (8-10 days old) began to swim in their characteristic undulating fashion in a straight line towards the window of the chamber. After contacting the window, they moved back and forth in the immediate vicinity of the window. They did not habituate during the course of 10 minute experiments. Using the Chi-square test for goodness of fit to a uniform

Figure 30. 9-10 day larvae stained with neutral red. (30a) Stained neural processes are seen in the larval episphere at the upper and lower edges of the prototroch. Although the upper nerve passes by the eyespots, no direct connection between the two structures is seen. (30b) The outer rim of each of the 2 lobes of the cerebral ganglion is stained very clearly. Part of the posterior prototrochal nerve ring is stained. (30c) Dark staining cells or groups of cells are found in the posterior region of the body.

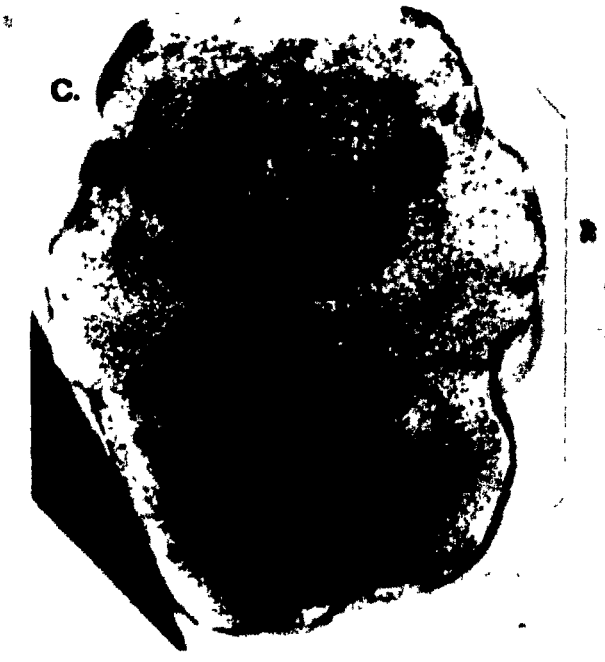




Fig. 30

Figure 31. (a) Blue-green fluorescence in 9-10 day larvae in areas that correspond to catecholaminergic cells putatively identified with neutral red staining and ultrastructural study. X 640.

(b) Confirming the neutral red staining cerebral areas, anterior and posterior prototrochal nerve rings and brightly fluorescing bodies in the lower end of the gut. Eyespot (e), prototroch (pr) and gut (g). X 560.

(c) Control larvae did not fluorescence blue-green regions but did autofluoresce in the prototrochal cilia and the gut. This autofluorescence was also seen in experimental larvae. X 560.

Fig. 31

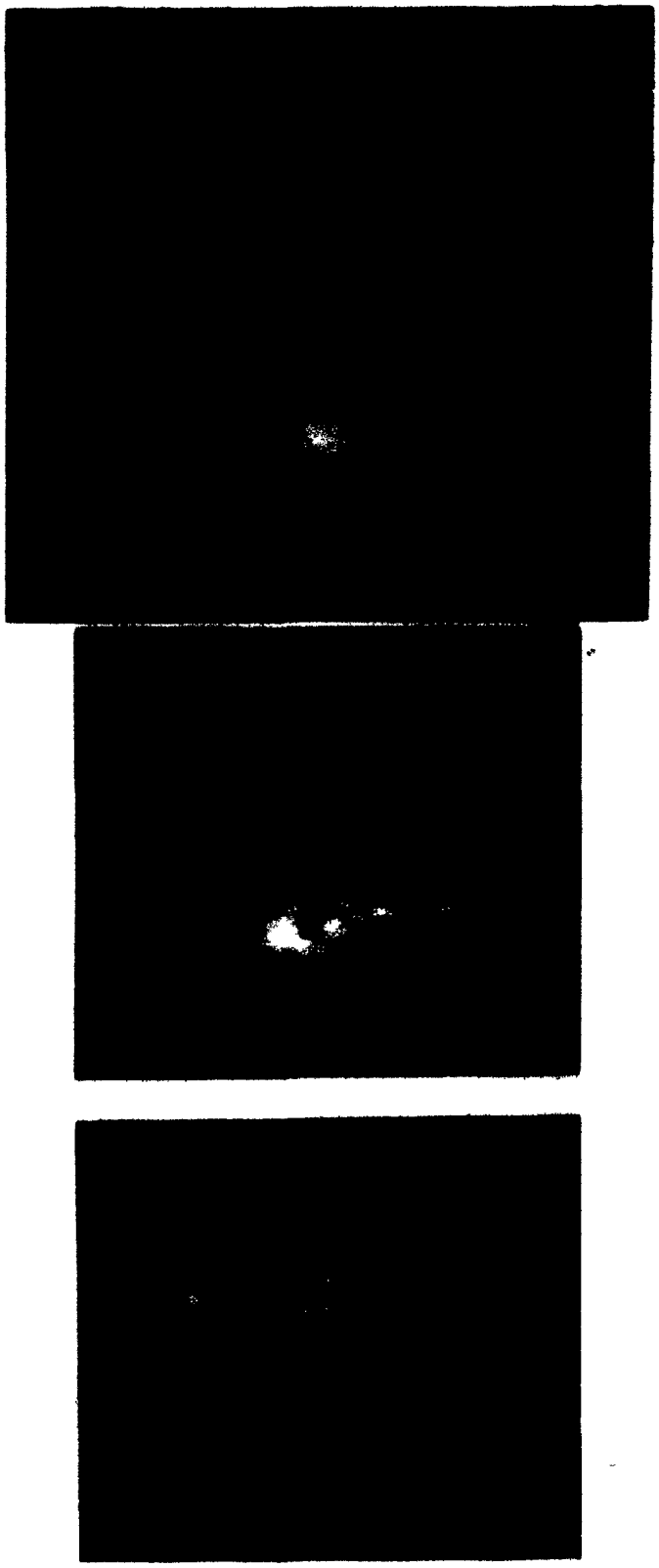
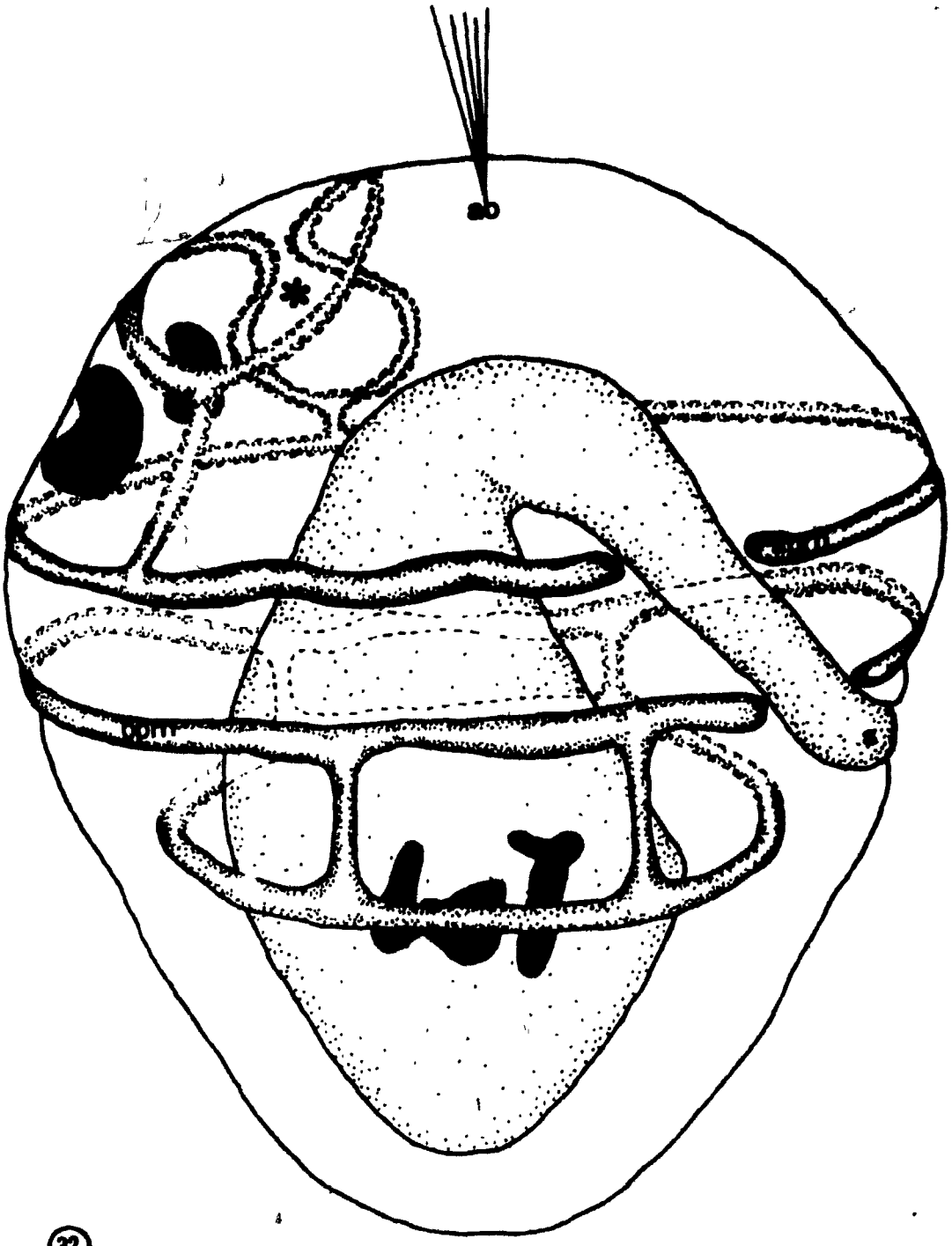


Figure 32. Distribution of catecholaminergic neurons in the 9-10 day larva; a summary of findings from ultrastructure, neutral red and glyoxylic acid studies. Eyes (e), cerebral ganglion (*), anterior (aprn) and posterior (pprn) prototrochal nerve rings and cells or groups of cells in the post-trochal region. Stomodaeum (s), apical tuft (ao).



(32)

distribution (Adler and Roessler, 1968) the larvae at position A, B and C in the chamber in white light were found to be asymmetrically distributed ($p < 0.001$). This asymmetry was also evident in experiments using red, green and blue filters but was only marginally significant ($p < 0.2$) during the first trial in red light. Larvae dark-adapted for 30 minutes, were uniformly distributed, a condition which was not disturbed by the flash of the camera. Figure 33a,b,c,d,e and Table II summarize these results.

Figure 33. Light responses of 9-10 day larvae at levels A,B and C. (a) Histogram of white light; (b) red light; (c) green light; (d) blue light; (e) 30 minute dark adaptation and flash effect.

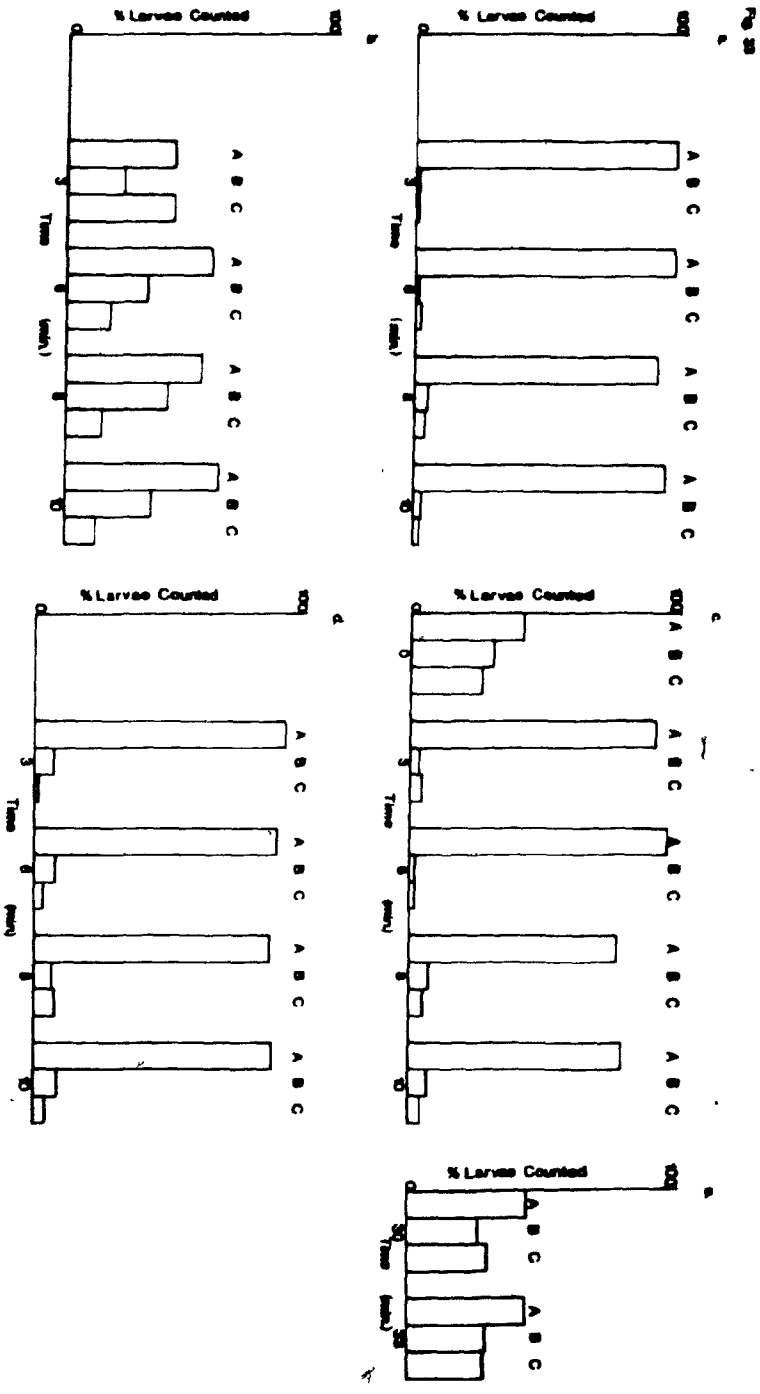

% Larvae Counted
Time (min)
A B C

Table II.

| Time (min) | Light Source | | | | |
|---|---|---|---|---|---|
| | White | Red | Green | Blue | Dark Adapted |
| 0 | --- | --- | P 0.200 | --- | --- |
| 3 | P 0.001 | P 0.020 | P 0.001 | P 0.001 | --- |
| 6 | P 0.001 | P 0.001 | P 0.001 | P 0.001 | --- |
| 8 | P 0.001 | P 0.001 | P 0.001 | P 0.001 | --- |
| 10 | P 0.001 | P 0.001 | P 0.001 | P 0.001 | --- |
| 30 | --- | --- | --- | --- | P 0.10 |
| 33 | --- | --- | --- | --- | P 0.10 |

## Discussion

In all species of polychaetes the pattern of cleavage up to the 64 cell stage is the same. Each cell occupies the same spot in relation to other cells. Subsequently, groups of cells begin to segregate and give rise to a blastula wall made up of clearly defined presumptive areas (Anderson, 1966). These areas may differ from species to species with respect to the number and identity of the cells which segregate into them. In general, however, the anterior daughter cells of the first quartette ($1a^1$ $1d^1$) divide to form a cap of cells which will fill the area anterior to the presumptive prototroch. The most anterior of these cells form an apical rosette which gives rise to the apical tuft. The tuft can vary in extent, number and origin of cells and may in some species be absent. Harmothöe imbricata has 4 cells making up its apical organ. As Lacalli (1981a) has discussed, the function of the apical tuft and its surrounding plexus in larvae of other species is not well understood. Only one cell in H. imbricata is ciliated and its many mitochondria suggest a high level of activity. Although the neuropil consistently develops beneath the cells of the apical organ, there is no evidence suggesting a neural connection between the two.

There is a striking similarity in the cytoplasm of the apical cells of Harmothöe imbricata and Phyllodoce maculata (Lacalli, 1981a). Apical cells in both species have an extensive Golgi apparatus and a patchy appearance due probably to large vesicles rupturing during fixation. Perhaps this similarity is suggestive of a common lineage for apical ciliated cells in these two families of polychaetes.

(polynoids and phyllodocids). However, unpublished results (Marsden) with serpulid larvae suggest that the apical cell cytoplasm in this family does not resemble that in polynoids and phyllodocids.

Generally, the apical rosette cells ($1a^{111}$-$1d^{111}$), and peripheral cells $1a^{112}$-$1d^{112}$, $1a^{121}$-$1d^{121}$ and $1a^{122}$-$1d^{122}$ divide further to form the cerebral ganglion and surface epithelium; collectively known as the anterior ectoderm of the trochophore. In all species, the presumptive prototroch is a result of proliferation of posterior daughter cells of the first quartette ($1a^{211}$-$1d^{211}$, $1a^{212}$-$1d^{212}$, $1a^{221}$-$1d^{221}$ and $1a^{222}$-$1d^{222}$) and various surrounding blastomeres (Anderson, 1973). In H. imbricata these presumptive cells develop in situ into 16 large ciliated cells arranged as 8 cells in each of 2 rows. The ventrally located daughter cells of the second and third quartettes give rise to the stomodaeum. By invagination these cells sink in the midventral line below the presumptive midgut and surround the anterior face of the gut mass. The stomodaeal rudiment becomes a distinct tube enclosing a lumen that maintains the mouth as a superficial aperture. Continual proliferation, of the stomodaeal rudiment from day 7 to day 10 in H. imbricata larvae, ends with the appearance of a connection between the stomodaeal lumen and that of the endodermal gut mass. At this time, however, the proctodaeal opening is not yet apparent. To compensate for the inward movement of cells in the ventral region, the posterior ectoderm stretches both posteriorly and laterally.

It has been suggested in other studies on the behaviour and morphology of polychaete larvae that functional nervous connections

appear only at the time when the larvae have achieved competency for metamorphosis. In larvae of the polychaetes, Arenicola cristata, Nereis virens and Capitella capitata, such neural connections are limited to those structures which persist in the adult, such as segmental musculature (Marsden and Lacalli, 1978; Bell, 1980; Lacalli, 1981a) and the paired eyes (Bhup and Marsden, 1982).

There is, however, also evidence for neural connections with larval structures in some serpulid and phyllodocid larvae (Lacalli, 1981a and Marsden, 1982). Lacalli describes a system of neurites arising from apical cells in S. polycerus and Phyllodoce minuta which connect with the eyespot and possibly the prototroch. Neurite-like extensions from prototroch-neurotroch cells have been seen in early larvae of Galeolaria caespitosa (Marsden, 1982).

In the larvae of H. imbricata morphological features which are strictly larval in nature are the apical organ, the 2-celled eyespots and the prototroch. Ultrastructural study of the apical region has revealed no connection between the apical organ and the neurites serving the eyespots and prototroch, as is probably the case in trochophores of S. polycerus and P. minuta (Lacalli, 1981a). Rather, the prototrochal nerve ring appears to be continuous with two dorsolateral connectives which extend ventrolaterally from the paired cerebral masses. Within each cerebral mass is a rhabdomeric eyespot and an axon extending towards the prototroch. The prototrochal nerve appears for the first time between day 6 and 7 of development. It consists of about five nerve processes, in the blastocoelic region of the prototroch, carrying large lucent vesicles and a few scattered

dense core granules. No signs of synaptic specializations were obvious at this stage. The complexity of this nerve increases along with the proliferation of the cerebral masses to form the cerebral ganglion. Although components such as synapse-like specializations between nerve fibers and cells of the prototroch and muscle cells, were not seen until day 10, some effective connections may well have been present earlier since responses to light and to gentle mechanical stimulation to the larval mid-region are evident by day 9.

Muscle fibers are found in the blastocoel of the episphere and also, accompanied by nerve processes, at a muscle insertion site in the stomodaeal epithelium. The nerve involved appears to be a branch of the prototrochal nerve. Lucent vesicles in these processes have been observed to form synapse-like clusters, presynaptic to prototrochal cells, as well as omega profiles on plasma membrane adjacent to muscle membrane. The possibility that the same group of nerve fibers serves both prototrochal and muscle cells could explain the observation that ciliary beating becomes slower immediately before the onset of induced muscular contractions. Contraction of the muscles around the stomodaeum would presumably change the shape of the stomodaeum and might also effect the slight mid-regional contractions seen on day 9. Stomodaeal muscle is unlikely, however, to account for changes in overall body shape. As mentioned previously, the ultrastructure study reported here was restricted to the episphere. Consequently muscle cells in the hyposphere were only putatively identified at the light microscope level. In 10-day larvae extended mesenchymal cells with nuclei located near the posterior end of the

body were seen to pass anteriorly along the body wall. Sites of insertion could not however be determined and require further study at the electron microscope level. Contraction of such muscle could effect the antero-posterior shortening response to touch seen on day 10.

The clusters of small lucent vesicles look very much like traditional synaptic clusters. These are usually seen adjacent to membrane specializations; the occasional cluster found at some distance from such terminals, probably represents the peripheral portion of a larger cluster at a release site, since the area of contact between a cluster and site of release is usually smaller than the area of cross section of the cluster (Gray, 1966; May and Golding, 1982a,b). None of these clustered vesicles were observed to exocytose as they have been seen to do in adult worms of the same species (May and Golding, 1982a,b). On the other hand, individual vesicles were observed to form omega profiles on the plasma membrane of nerve processes adjacent to muscle inserted on the stomodaeum. The coated vesicles occassionally seen in prototrochal nerve processes presumably represent endocytosis. The coincident appearance at day 10 of both endo and exocytotic profiles suggests functional synaptic activity in H. imbricata larvae by the 10th day of development. The clusters of small, lucent vesicles and the cleft (20 nm) between 2 membrane specializations are always associated with a dense matrix which has also been seen in synapse-like structures in adult worms (May and Golding, 1982a,b) although its significance is unknown.

Of the two remaining populations of vesicles/granules, neither

appear to be directly involved in the clusters. Dense-corded granules (65-100 nm) similar to those found concentrated in the neuropil and scattered in prototrochal nerve fibers of the larvae of H. imbricata are known to be associated with the neurosecretory complex of adult worms (May and Golding, 1982c). Frequent dense-cored granule exocytosis has been found in the adult neuropil but has not been seen in the larvae of H. imbricata. However, the similarity of morphology of these granules in both stages of the life cycle raises the possibility that they may serve a hormonal function in larvae as well as adults.

Three types of processes have been identified in the nervous tissue of 6 to 10 day H. imbricata larvae. The electron-lucent prototrochal nerve processes contain all three types of vesicles/granules, innervate prototrochal and muscle cells, follow the same developmental time frame as the cerebral cells and thus could possibly arise from the cerebral cells. The fact that neurites have been seen leaving the eye and other cerebral cells to travel as a bundle posteriorly towards the prototroch and the observation that all other cells under the prototroch are medial to the basal lamina, together support the notion of a cerebral origin for the prototrochal nerve. The second type of nerve process is unique and confined to the neuropil. These electron-dense processes have a characteristically high content of dense cored granules. They are present along with electron-lucent processes in the cerebral neuropil at its earliest stage of development and persists throughout all the stages of larvae used in this study. Processes of this sort have not been seen in the

prototrochal nerve. Finally, a third morphologically distinct type of nerve fiber appears on day 10 of development. It resembles, in cytoplasmic morphology, prototrochal cells but, in contrast to non-neural prototrochal basallar folds, this third type of fiber contains the 3 vesicle/granule types characteristic of the lucent processes, although synapse-like specializations were not seen. These processes intermingle with prototrochal nerve fibers, prototrochal basallar folds and muscle. It is not clear whether this third fiber type represents a selective maturation of certain prototrochal nerve processes or an invasion of the prototrochal nerve by a new kind of cell.

Neutral red, which is selectively taken up by aminergic cells (Stuart, et al., 1974), clearly stained the cerebral masses, a double prototrochal ring, a network of processes in the episphere and a few large cells in the posterior part of the body. The stained ring anterior to the prototroch did not appear to have a connection to the posterior stained ring. Although staining appeared around the eyes, no evidence for direct contact between the eye and nerve in the episphere was apparent. Glyoxlic acid experiments yielded results which appear to demonstrate the same system, presumably a catecholaminergic component of the nervous system which corresponds, at least in part, with the distribution of dense-cored granule-containing neurites seen in the ultrastructural study. Although the glyoxylic acid method provides a simple technique for the localization of catecholamines and their precursors, it does not provide for the spectral differentiation of secondary catecholamines (eg. epinephrine)

from primary catecholamines (eg. dopamine). Histochemical fluorescence microscopy has other limitations also; fluorescence intensity of catecholamine fluorophores is not proportional to amine concentration over a very wide range and thus is not quantitatively useful (Occur and Berlund, 1983). However these disadvantages should not overshadow the fact that this type of technique has made possible, extensive mapping of monoaminergic pathways in vertebrates as well as in invertebrates. These above two techniques also served to reveal catecholaminergic areas of the hyposphere of the larva, a region not explored ultrastructurally in this study.

The cerebral ganglion begins to form from two cerebral masses appearing dorsolaterally between day 6 and 7 of development. The two masses precede by one day the appearance of a pair of bilaterally, symmetrical eyes located at the surface of the central area of the cerebral masses. Cephalic photoreceptors of either the inverse pigment cup type or the inverse epidermal type are characteristic of polychaete worms. The inverse pigment cup type of the polychaete adult and larval ocellus is claimed by Salvini-Plawen and Mayr (1977) to be devoid of ciliary structures. H. imbricata larval eyespots conform to this generality. Following the appearance of the eyespots, a neuropil (the rudiment of the cerebral commissure) begins to form beneath the apical organ. A bilobed cerebral ganglion occupies most of the dorsal-anterior ectoderm of the 10 day larva. Neutral red staining confirms the position of the two lobes of the cerebral ganglion and also the latter's connection with the prototrochal nerve.

As described, neutral red does not reveal a direct connection

between the larval eyespots and the prototrochal nerve. However, structurally, a neurite is seen to arise from the receptor cell and to travel with other cerebral cell neurites towards the prototrochal region. In addition, a positive light response indicates some photoreceptive control of swimming. Once an impulse is generated, it seems probable that it could be carried via the receptor cell axon to the prototrochal nerve where it could then modify the activity of prototrochal cilia. Orientiation by H. imbricata could be a matter of balancing the stimulation received by the 2 eyespots, unlike the situation in single-eyed trochophores of S. giganteus and S. vermicularis.

The positive light response of H. imbricata larvae is persistent, like that of Spirobranchus giganteus trochophores (Marsden, in preparation) and unlike that of Serpula vermicularis larvae which habituate within a few minutes (Young and Chia, 1982). The spectral sensitivity (within the range of visible light) of all three species is similar, although H. imbricata appears to be more responsive to light of wavelengths longer than 580 nm. However, this apparent difference needs to be substantiated by further study. Identities of the neurotransmitter used by photoreceptors are not known in any invertebrate retina although in the cephalopods acetylcholine and dopamine are two possible candidates (Lam, et al., 1982). The observation that neutral red and glyoxylic acid do not stain the receptor cell neurite suggests the possibility that a non-catecholaminergic transmitter, may be responsible for the photosensory mediated behaviours of H. imbricata larvae.

The larval polychaete eyespot has been described for Neanthes sucinae (Eakin and Westfall, 1964) and S. giganteus (Marsden, in preparation) as well as H. imbricata (Holborow and Laverack, 1971). Both of the first 2 accounts indicate a structure similar to, but smaller than the eyespots of H. imbricata larvae. Nothing is known about the comparative roles of the evaginated and invaginated membranes of the sensory cell or about the significance of variations in size, or number of microvilli and very little about the role of these interesting structures in the behavioural repertoire of the larva.

The trochophore larva of Harmothöe imbricata appears to utilize a simple nervous system which includes early stages of the developing adult nervous system. If natural release from parental protection takes place at about 14 days it must happen after the establishment of connections between the cerebral ganglion, including the eyespot, and the prototrochal ciliated cells as well as muscle. Although capable of development in the absence of parental protection, the latter is presumably beneficial since these larvae are not capable of feeding until the end of or after the 14 day brooding period. In summary, therefore, these larvae are normally released when they are able to feed, are equipped with a nervous system connecting eyespots, cilia, muscle and possibly the apical tuft, and can react to light and probably other features of their natural environment.

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