EARLY DEVELOPMENT OF THE THORAX

AND THE NERVOUS SYSTEM

OF THE BRINE SHRIMP ARTEMIA

A thesis submitted for the degree of

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by

CLARE ELIZABETH BLANCHARD

Department of Zoology,

University of Leicester

Leicester LE1 7RH.

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DEDICATION

To my parents

Abstract

Early development of the thorax and the nervous system of the brine shrimp *Artemia*

by

Clare Elizabeth Blanchard

The early development of the thorax and the nervous system of *Artemia* is described and compared with that of other arthropods.

The thoracic segments of *Artemia* develop in an antero-posterior sequence, as pairs of mesoderm bands are segregated off from the growth zone in the posterior of the larva. These mesoderm bands, with their associated ectoderm grow and differentiate gradually, each segment eventually bears a pair of ventral appendages called phyllopods, and a pair of mechanoreceptive setae dorsally. Seven stages are designated in a description of the external development of the segment, with specific reference to the morphogenesis of the phyllopods.

Two pairs of terminal pioneer neurons located near the posterior tip of the larva are described. Their axons grow out over uninnervated tissue to pioneer firstly the longitudinal connective pathways. These axons then branch medially in an antero-posterior sequence as segment development progresses to pioneer the commissures. The posterior commisure pathway is completed before the anterior one in each segment. The two dorsal nerves in the posterior of each segment are the first peripheral nerves to be pioneered to the C.N.S., by axons from the pairs of neurons that innervate each dorsal seta.

Ultrastructural descriptions are given of the terminal and dorsal pioneer neuron cell bodies, axons and associated structures. The dorsal seta neurons have a sensory as well as a pioneering function, and the terminal pioneer neurons may also be derived from the neurons of sensory receptors. The ultrastructure of the dorsal setae is uniquely simple compared to other crustacean mechanoreceptors. In the ventral cord the location of the early pioneer neuron cell bodies and the pathways pioneered by their axons are different and far less complex than in decapod crustacea or insects.

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STATEMENT

The accompanying thesis submitted for the degree of Ph.D. entitled 'Early development of the thorax and the nervous system of the brine shrimp Artemia' is based on work conducted by the author in the Department of Zoology in the University of Leicester during the period between October 1980 and October 1983.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

Signed ..C.E.Blanchard..... Date ....7/3/86....

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CHAPTER ONE

INTRODUCTION

Development of the nervous system

The study of the formation of the nervous system has been one of the most challenging investigations undertaken by developmental biologists. The nervous system of even quite primitive invertebrates is an extraordinarily complex assembly of interconnected cells, designed to fulfil the central role of co-ordinated information transfer within the body. The structure of the nervous system provides a framework which has evolved to optimise the efficiency of information flow, at the same time as maintaining a level of plasticity within the system to allow some degree of functional survival of perturbations and damage during development. Much information has been gained in recent years about the cellular mechanisms involved in the building of relatively simple invertebrate nervous systems.

The research presented in this thesis is mainly concerned with the early neural development of a simple crustacean; the brine shrimp Artemia. This provides a useful comparison with the detailed insect studies and demonstrates that there is more than one way of forming the framework of the basic ladder-like arthropodan nervous system. Developmental studies are increasingly contributing to our understanding of the evolutionary

connections between different groups of animals (Anderson, 1973), and in this respect Artemia is a particularly significant animal to study from among the Crustacea as it is phylogenetically primitive.

The development of the nervous system in the thoracic segments was specifically chosen for investigation for a number of reasons. The eleven thoracic segments and their associated ganglia all develop after the nauplius larva has hatched from the egg. These segments arise and differentiate progressively in an antero-posterior sequence; eventually each will bear a pair of appendages called phyllopods. A consequence of this order of development is that in some of the early instars a single larva will show the complete range of stages in thoracic segment development, from mature phyllopod-bearing segments anteriorly to newly emerged, smooth segments posteriorly. Therefore, information about development of the nervous system at different stages of thoracic segment development can be gathered from analysis of a single specimen.

A description of thoracic segment development and phyllopod morphogenesis was also undertaken, in order to relate fully the early development of the nervous system to the development of the segment as a whole.

As an introduction to the two aspects of this research there now follows firstly a brief account of embryonic and segmental development in Crustacea including Artemia. The second part of the Introduction is a comprehensive review of the literature concerning formation of the framework of the arthropodan (usually insect) nervous system, followed by

a review of previous research on or related to the Artemia nervous system.

Development of Crustacea

Embryonic development

Information concerning the embryology of arthropods has converged in recent years to support the view that the arthropods are a polyphyletic assemblage (Anderson, 1973). The Crustacea are one of the three major groups of living arthropods and are the most diversified in form. The other two groups are the Uniramia and the Chelicerata. Each group has an underlying unity of embryonic development and shows a unique pattern of ontogeny which could enable each group to be considered as a phylum.

Although embryonic development and adult morphology of the Crustacea is highly variable among and within classes, there are fundamental unifying similarities which characterise the phylum. Crustacean embryonic development involves relatively superficial cleavage due to the presence of large amounts of yolk, and passes through a blastula stage which exhibits a characteristic fate map of areas of cells with particular developmental fates. The blastula then develops initially into the nauplius larva and proceeds through a series of moults to the adult form. A nauplius larva is one whose propulsive appendages are limited to the 1st antennae, 2nd antennae and the mandibles. The two pairs of antennae are a diagnostic feature of all Crustacea.

Segmental development

Development then continues by progressive formation of post-naupliar segments from a pre-telsonic growth zone. The process by which these segments are formed and added is one that is basically similar to that observed in Annelida with a teloblastic origin of mesoderm bands. Artemia probably exhibits a primitive mode of this development, with a slow addition of segments through many moult stages. The development of the thoracic appendages occurs after segments are first recognisable (Anderson, 1967). In Crustacea there are groups of segments, the tagmata, which are structurally marked off from other groups and are specialised to perform certain functions for the whole organism. This may result in fusion, loss or modification of somites which will obscure the regularity of repetition of certain internal structures or of the serial succession of segments themselves. The original pattern of segments can often be assessed more easily at the embryonic stages than in the mature adults. From embryological and developmental studies of many different Crustacea, a pattern of segmentation and organisation of segments has been determined which is exhibited by even the most diversely adapted forms. The crustacean body is divided into a head, thorax and abdomen. The head has five obvious segments (but six embryologically), all of which bear appendages. Typically, the thorax is composed of eight segments, all bearing appendages, although there are thirteen in Artemia (including two genital segments). The crustacean abdomen is typically six - segmented (with or without appendages) but in Artemia there are six abdominal segments and a terminal telson.

Formation of the nervous system in Arthropods

The nervous system of vertebrates and most invertebrates is composed of central and peripheral components. The peripheral nervous system (P.N.S.) consists of bundles of axons which form the peripheral nerves connecting all parts of the body, and it also includes the peripherally located sensory neuron cell bodies (some of which may be organised into small peripheral ganglia such as those associated with the gut in insects). The C.N.S. contains many cell bodies (motorneurons and interneurons) and most of the synapses between neurons, it is thus the location of most neural information processing. Axons within the C.N.S. are co-ordinated together into tracts, commissures or connectives, all of which are formed by the association of axons. It is clear from this description that there are major routes (eg. nerves, tracts) that are followed by large numbers of axons to guide them all to a particular region, but each neuron must then make the highly specific connections that are particular to itself.

Neural development in a variety of arthropods has been studied intensively in the past twenty years or so, using a wide selection of analytical techniques to try and determine some of the factors involved in the construction of these relatively simple nervous systems. These include a variety of methods for visualising neurons and their processes, such as fluorescent dye injection and labelling with horseradish peroxidase or monoclonal antibodies. Genetic variants affecting neural development or formation of the basic body plan (eg. homeotic mutants), have provided an experimental tool for disrupting an animal's development non-

surgically and have been particularly valuable in determining the effects on neurons of misrouting them from their normal paths. Serial sectioning followed by light or electron microscopy has allowed detailed reconstruction of neuronal anatomy in many invertebrates and has also been usefully combined with histochemical staining procedures. The majority of these studies have been investigations into the development of insect nervous systems.

It is now generally accepted that there is no single guidance system in operation but that a variety of mechanisms are employed in the construction of the arthropod nervous system. The main results of these studies will now be described.

Pioneer neurons in insects

The development of the nervous system of many arthropods has been analysed down to the level of single cells. Of particular interest has been the description of individual neurons which send out axons early in development over uninnervated tissue, providing guidance for subsequently differentiating neurons. Bate (1976) was the first to describe 'pioneer neurons' in the developing insect nervous system. He detected single pairs of axons at the base of the antennae and limb buds during embryonic development of the locust. They are derived from pairs of peripheral neurons lying at the distal ends of these embryonic appendages and ultimately the pioneer growth cones enter the embryonic neuropile (Keshishian, 1980). (The growth cones are flattened amoeboid processes at

the end of many extending axons (Cajal, 1960; Harrison, 1910)). The neurons thus establish pathways between the periphery and the C.N.S. very early in development when distances are short and appendages have not elongated. These pathways subsequently become the routes of major nerves as further axons join the axon pairs (Bentley and Keshishian, 1982a). Pioneer neurons have now been described in all segments of grasshopper metathoracic legs (Bentley and Keshishian, 1982b), in the cerci of the grasshopper (Shankland, 1981) and of the cricket (Edwards and Chen, 1979). Pioneer neurons are not only involved in the formation of the P.N.S. but have also been shown to form the early ladder-like framework of the C.N.S. in the grasshopper embryo (Bate and Grunewald, 1981; Goodman et al., 1984).

Insect guidepost cells

Detailed studies of the appearance of central and peripheral pioneer neurons in the grasshopper, and the courses followed by their growth cones, have indicated the dependence of some of these pathfinding axons on other neuronal cell bodies that appear to act as landmarks. The first growth cones in the metathoracic limb bud navigate along a chain of 'guidepost' cells to the C.N.S. (Goodman et al., 1982), establishing filopodial contacts and dye-coupling with these cells only (Taghert et al., 1982). After selective destruction of the pioneer neurons, Bentley and Keshishian (1982a) found that later differentiating neurons could still navigate the same path so long as the guidepost cells were available. It has also been shown that pioneer axons lose their directed out-growth if the guidepost cells are

selectively killed (Bentley and Caudy, 1983). This evidence indicates that the ability to pioneer future nerve pathways may not be unique to pioneer neurons. In their absence, other neurons may fulfil their role if the necessary guidance cues are still available, in this case it is the guidepost cells which specify the route. When the pioneer neurons at the tip of the cricket cercus are destroyed (Edwards et al., 1981), the neurons which arise subsequently near the base of the cercus still initiate axonogenesis and fasciculate together. They form however, an aberrant number of bundles instead of the normal two. It is possible that in this case the landmarks that would have been followed by the pioneers were not still available for later differentiating neurons. This could have been because by then development had progressed too far, so that the guidepost cells had either disappeared or become out of reach. Alternatively, these cues may have been damaged or destroyed during ablation of the pioneer neurons, or could not be recognised by neurons other than the pioneers.

Insect larval nerves

Another slightly different example of neuronal guidance by pre-existing axons is found in holometabolous insects, whose adult epidermis is derived from imaginal discs. Most, if not all discs have neural connections with the C.N.S. that are probably established during embryogenesis when distances are short (Bate, 1978). These larval connections provide guidance for the enormous numbers of axons from adult sensory neurons which appear at metamorphosis. This has been demonstrated for the antennae of the moth Manduca sexta (Sanes and Hildebrand, 1975;1976) and also for the

developing compound eye of the butterfly Danaus plexippus, (Nordlander and Edwards, 1969) where the stemmatal nerve of the rudimentary larval eye is followed by adult optic axons.

Mis-routing of axons in homeotic mutants of Drosophila

The guidance cues that are followed by axons along peripheral routes such as these have been shown to be navigable by ingrowing sensory axons other than those normally following these routes. Experimental mis-routing of sensory axons in Drosophila by surgical or genetic techniques causes them to enter the fused thoracico-abdominal ganglion via abnormal paths and yet they can form morphologically (Ghysen, 1978; Anderson and Bacon, 1979) and functionally (Stocker, 1977; Vandervorst and Ghysen, 1980) normal projections. This indicates that there are sufficient homologous guidance cues in both the C.N.S. and P.N.S. of Drosophila to allow correct connectivity to be established by some neurons which stray outside their normal paths and enter the ganglion via the wrong nerve. This result is not achieved if the neuron is displaced too far, and it enters the wrong ganglion. What happens in those cases is discussed later. The only choice involved in an axon following a particular peripheral path to the C.N.S is that it appears to select the closest one available. The formation of multiple nerve bundles in the cricket cercus after ablation of the pioneer neurons is another example of this phenomenon, where axons adhere to and follow any axons they encounter. The order in which axons join a bundle, and the number of axons within a bundle, are clearly not rigidly determined. This tendency for axons within a region to fasciculate

together was shown many years ago in the bug Rhodnius by Wigglesworth (1953). If the normal nerves in the epidermis are destroyed, the axons of newly-generated sensory neurons always tend to associate into bundles, sometimes forming highly abnormal circular whorls of axons. A comparable effect is observed in the Drosophila mutant extra eye where the axons from the supernumerary eye generated in this mutant seldom innervate the brain. The axons generally form large fasciculated whorls within a tissue mass of muscle and tracheae underlying the ectopic ommatidia (Marcey and Stark, 1985). It is this affinity of axons for each other that makes the pioneer neuron system such an effective mechanism for guidance of large numbers of neurons towards a target region such as the C.N.S.

Polarity information in the insect P.N.S.

There must be some indicator of proximodistal polarity to which the emerging growth cones of pioneer and other neurons in embryonic appendages can respond. These growth cones grow proximally towards the C.N.S. and they are not directed that way by guidepost cells. The neurons which pioneer the nerves in grasshopper antennae have been shown to grow along the epithelial cell surface, with their filopodia in direct contact with these cells. Berlot and Goodman (1984) have proposed that there is an adhesive gradient of surface molecules on the epithelium which causes the growth cones to grow proximally, and that they are only deflected from this course when they are within filopodial grasp of the guidepost cells. They suggested that the surfaces of the appropriate guidepost neurons are

more adhesive for the pioneer neurons than the epithelium, and that guidance to the C.N.S. is achieved by an 'adhesive hierarchy', where a proximal guidepost cell is the most adhesive for a neuronal growth cone, and distal epidermis is the least adhesive surface. Two different investigations into neuronal pathfinding in insect wings have demonstrated that the polarity information in these cases is also located in non-neural tissue. New sensory neurons which arise in the metamorphosing wing of the moth Manduca sexta always grow proximally and are normally guided by the pupal neural network already present in the wing (and presumably laid down by pioneer neurons). If, however, this pattern is disrupted by epithelial grafting of proximal tissue distally and vice-versa, predictable changes in axon growth patterns occur. These new growth patterns are not caused by newly-formed nerve patterns, but are explicable in terms of position-specific properties of the wing epithelium or perhaps its basal lamina, which express a proximo-distal polarity (Nardi, 1983). (The basal lamina is an extracellular matrix secreted by epidermal and neural ectoderm which separates it from the mesoderm, it is equivalent to the basement membrane, a term more commonly used in vertebrate anatomy). Neuronal navigation is shown once again to be potentially independent of pre-existing nerves or pioneer pathways, for in their absence, axonal growth cones in Manduca sexta wing continue to grow proximally, guided only by polarity information in the substrate over which they are growing. A comparable study has been made of the polarity of axon growth in the wings of the mutants Hairy wing and hairy of Drosophila (Palka et al., 1983). The supernumerary sensory axons that are generated in these mutants only reliably grow towards the base of the wing if they encounter

either of the two normal nerves within wing veins L1 and L3. Axons developing elsewhere in the wing have even been observed to grow proximo-distally. An analysis of the formation of these nerves has shown that they are formed in sections with specific neurons pioneering defined stretches of the pathways within the wing veins (Murray et al., 1984). The TSM (1) cell pioneers the proximal portion of the L1 nerve to the base of the wing, it is not observed to contact any guidepost cells but simply grows proximally in vein L1. The distal part of this nerve is formed by bristle neurons along the anterior edge of the wing. These cells are so close to each other that their cell bodies are joined together in the form of a chain by the fasciculation of their axons. The axons all grow proximally along wing vein L1 and join the proximal path formed by TSM (1). This is an example of quite simple neuronal guidance where the basic requirement is to direct axons proximally to the base of the wing. There do not appear to be any guidepost cells and instead, the wing veins supply physical channels for axons and also disto-proximal polarity information. An absolute necessity for neurons to pioneer the proximal part of the nerve has not yet been proven, and it may be that the non-neural cues would suffice to guide any neuron.

Polarity information in the insect C.N.S.

Pioneering neurons in the grasshopper C.N.S. appear to have access to polarity information in the antero-posterior direction, perhaps located in the basal lamina, which forms an integral part of the landscape over which these neurons navigate. The C.N.S. of the grasshopper forms from the

neural embryonic ectoderm as a longitudinal strip of cells which runs down the middle of the embryo from head to tail. The dorsal surface of this neural epithelium is covered by a basal lamina across which the central pióneers navigate (Bate and Grunewald, 1981). Although this phenomenon could be the inevitable result of axons emerging into an environment bounded by a basal lamina, experimental evidence shows that growth cones do actively seek this substrate. The first central neurons that begin to pioneer in the thoracic segments are two pairs of sister cells either side of the ventral midline that are the progeny of a pair of midline precursor cells: the MP2 cells. The growth cones of each pair both travel a short distance dorsally to the basal lamina although one cell body lies in a ventral location beneath the other. The axon of the dorsal cell grows posteriorly and that of the ventral cell grows anteriorly along the basal lamina, to the segment borders. They then cross the borders and contact the equivalent axons in the adjoining thoracic segments, thus laying the path for the intersegmental connectives. The possible role of guidepost cells in this instance cannot be ruled out although sufficient guidance could perhaps be achieved if the basal lamina were polarised in the antero-posterior axis, and the sibling neurons had mirror-image properties in their response to this polarity. There are however, three conspicuous cell bodies in the posterior direction which are also involved in the early formation of axonal pathways and some or all of which could serve a guidance function for the posteriorly growing MP2 cell. (Goodman et al., 1982).

The interaction of a pioneer growth cone with a guidepost cell

The nature of the interaction of a pathfinding neuronal growth cone with one of the probable guidepost cells on its route, has been investigated using transmission electron microscopy by Bastiani and Goodman, (1984). They studied the interaction of another of the midline precursor cells in the C.N.S. of the grasshopper embryo : MP1, which grows posteriorly like the dorsal MP2 cell mentioned earlier, and helps form the intersegmental connective. The growth cone of this cell interacts very selectively with the growth cone of the posterior corner cell (PCC) which lies on its pathway. MP1 filopodia were observed inserted up to seven μm into the PCC and they sometimes reached the nucleus of the cell. Coated pits and vesicles were present in the membrane of the PCC at the tips of the majority of these insertions, but did not occur with filopodia contacting the cell body of the PCC rather than the growth cone.

Formation of specific synaptic connections

The mechanisms described so far have been concerned with the pre-synaptic guidance of the growth cone and provision of a simple system of tracts to and from the C.N.S. and between ganglia. The ways in which neurons reach and recognise the specific individual neurons and processes with which to synapse will now be considered. One of the simplest possible combinations of factors to produce neuronal connectivity has been demonstrated in the eye of the water flea Daphnia. A highly ordered retina-lamina projection is achieved as the result of a well-defined

temporal sequence of growth and migration of retinula cells which 'recruit' presumptive lamina cells into optic cartidges (Lo Presti et al., 1973). The eight photoreceptor axons of each ommatidium project towards the lamina ganglion as a fascicle, navigating along a palisade of glial guidepost cells in the midplane. There is a leading axon which first enters the developing lamina and interacts sequentially with five presumptive lamina cells. These then generate neurites to join the fascicle of retinula axons and form an optic cartridge. The new cartridge is displaced laterally as the next fascicle of retinula axons is growing along the glial guidepost cells to repeat the process. This is an interesting example of an efficient use of cues, where the same set of guidepost cells are followed sequentially by all retinula pioneer neurons. Ultra-violet induced deletion of groups of retinula cells before such outgrowth is always associated with the formation of a smaller lamina, indicating the inductive effect of ingrowing optic axons on lamina ganglion cell survival and differentiation (Macagno, 1978; 1979). Presumptive lamina cells that would have been contacted by axons from an ommatidium which is then deleted, do not degenerate, but are recruited into a cartridge by the next fascicle of retinula axons to grow into the lamina. Thus, the smaller lamina always results from the degeneration of presumptive lamina cells that were destined to join the last ommatidia, irrespective of which retinula cells were destroyed. Retinula cells are clearly not specified for particular lamina cells, and this is not necessary in a structure whose parts normally mature in a spatially and temporally ordered fashion. This is another example of organisation within the developing nervous system where the need for cell surface markers specific to individual cells is minimised, as was seen for axons

following peripheral nerve routes. However, such a simple spatio-temporal system is not suitable for newly-arriving axons in other parts of the C.N.S. and the growth cones of developing central neurons (motorneurons, interneurons), which are immersed in the axons, dendrites and cell bodies of earlier differentiating neurons. It is the nerve fibre tracts into which the neuropile is itself organised, which provide the next level of guidance for pathfinding neurons in the C.N.S.. The selection of a particular tract within the neuropile has been graphically demonstrated in the grasshopper. The growth cone of a cell called the G-cell has been observed as it emerges into the developing neuropile. The filopodia of this growth cone are observed to contact the surfaces of twenty five longitudinal fascicles that are present within the neuropile before selecting one to follow (Raper et al., 1983). Furthermore, while making this choice, the growth cone exhibits a specific affinity for two out of the four axons in this fascicle. (Bastiani and Goodman, 1984). If these two axons are specifically ablated, the G growth cone does not show an affinity for any other axons, and only grows a short distance (Raper et al., 1984). Studies of misrouted haltere neurons in Drosophila mutants indicate that these central fascicles are sufficiently labelled to ensure that a growth cone will reach the correct region within the ganglion, even if it has to travel posteriorly along a stretch which would have normally been followed anteriorly (Ghysen, 1978). Such unusual but correct guidance of axons in the reverse direction to that normally taken by them has also been shown for regenerating neurons from the cricket cercus. Some regenerating neurons send axon collaterals erroneously towards the anterior border of the ganglion, at which point the collateral reverses course and returns to arborize the normal projection

area (Murphey et al., 1981). Localised groups of sensory neurons are known to project into discrete regions of their target ganglion, such as the antennal glomerulus of the Drosophila brain, into which all antennal neurons normally project. In a study of the projection patterns of neurons from bristles or campaniform sensilla of the thorax of mutant and wild-type Drosophila, the general pathway followed in the C.N.S. depended on the type of sensory structure and compartment (see Crick and Lawrence, 1975) of origin (Ghysen, 1980). However, the detailed projection of a single neuron within such a sub-group was determined by its peripheral location on the antero-posterior axis of the thorax. In this way, sensory information from the two-dimensional epidermal surface is mapped into a central network of neuronal connections. This type of somatotopic map of mechanoafferent projections has also been clearly demonstrated by Murphey (1981) for the cricket cercus. There are however other examples, particularly in studies of the locust, where neither specificity due to type nor site of peripheral origin can sufficiently explain projection patterns in all cases (summarised in : Braünig et al., 1983). The level of resolution provided by cobalt or horseradish peroxidase filling of neurons (which is the standard technique used in these experiments) is limited, and in some instances is not adequate to elucidate the factors involved in neuronal specificity. There are also reservations that should be expressed when experiments involving homeotic mutants of Drosophila are considered and the C.N.S. itself is mutated as well as the appendage involved. In spite of such considerations, a good indication of the validity of any theory is given when consistent results are obtained through a number of different experimental approaches.

Homology of neural development between species and within a species

Recent studies of embryonic neural development in Drosophila have revealed that it is a miniature replica of the grasshopper or locust embryo in terms of its identified neurons, their growth cones and their selective fasciculation choices (Thomas et al., 1984). It is thus feasible now to combine the cellular and molecular genetic analysis of cell recognition during neuronal development in Drosophila and to perhaps overcome the shortcomings of either approach when used in isolation. This striking similarity of embryonic neural development seen in Drosophila and the grasshopper has also been found to include the embryo of the crayfish Procambarus (Thomas et al., 1984). Homology is not only observed between species but is also found between the different ganglia of a single nervous system. There appears to be some degree of overlap and homology of neuronal guidance mechanisms between different ganglia in insects. This being yet another manifestation of the well-documented theory that the metamerically repeated segments of an insect have different functions and appendages that have evolved through variation of a primitive unspecialised segment, and that this common ancestry is sometimes revealed. As mentioned earlier, if a neuron is genetically misrouted into a completely different ganglion, it will not grow into its appropriate ganglion. It will, however, still form a complex projection in the ganglion entered. In a study of the mutant proboscipedia of Drosophila, where parts of the proboscis are transformed into leg and antennal tissue, the homeotic tarsal and aristal neurons both projected into the antennal and proboscis centres of the brain (Stocker, 1982). The

projection patterns of leg and antennal neurons which were misrouted into the proboscis centre are completely different from the wild-type proboscis projection and are very similar to each other. This demonstrates that the proboscis centre provides a sufficiently homologous environment compared to the usual thoracic ganglion or antennal centre, to allow connectivity to occur, and that this centre may be revealing some common properties of leg and antennal neurons. An underlying serial homology of neural primordia is seen in the grasshopper, whose thoracic and abdominal ganglia come from identical sets of embryonic precursor cells, although there are striking differences in the number and properties of neurons in the different mature ganglia (Bate, 1976; Bate and Grunewald, 1981). The fate of the progeny of a particular cell : Midline Precursor cell 3, has been examined as development progresses in all the thoracic and abdominal ganglia of the grasshopper (Bate et al., 1981). It was found that the two progeny of this same precursor cell in different segments can either live or die, and if they survive, can develop different morphological and physiological properties. These differences may be intrinsic to the cells in different segments or may result from initially identical cells being influenced by their segmental environment. Alternatively, a mixture of both factors may be important because the formation of a nervous system often involves interactive processes.

Cell-surface antigens

Throughout this discussion it has been assumed that there are specific markers, either on the surfaces of cells, nerves or the extracellular matrix,

which developing growth cones are preferentially attracted to and adhere to. Very little is known about these 'labels' but there are some studies which have revealed different antigenic and morphological properties of surfaces within the environment of developing growth cones. One hundred and forty-eight monoclonal antibodies have been raised against the Drosophila nervous system that exhibit anatomical specificity (Fujita et al., 1982). Antibodies were found specific to the neuropile or cortex of ganglia and to nerve fibres. The concentration of one such antibody was observed during metamorphosis in Drosophila, where there is extensive synapse formation in the neuropile of the ganglia with new imaginal cells differentiating after the breakdown of the larval neuropile (White et al., 1983). The concentration of this particular antigen, which is localised in the neuropile appeared to parallel the developmental process, with an initial decrease in concentration followed by an increase which may be correlated with the degeneration of the larval neuropile and the formation of the adult neuropile. An antibody raised against horseradish peroxidase recognises neuronal membranes in Drosophila and the grasshopper, including isolated pioneer neurons (Jan and Jan, 1982). There is evidently a neural antigen appearing early in the differentiation of the nervous system that is recognised by this antibody and is common to many neuronal cells and processes. It may be this label that causes peripheral axons to tend to fasciculate together non-specifically. Another monoclonal antibody(I-5) has been produced by Chang et al. (1981), which differentially recognises a variety of pioneer neurons and early axonal pathways in the grasshopper embryo (Goodman et al., 1982). The basal lamina of the moth wing has been investigated using scanning electron microsopy, which has revealed a

graded distribution of extracellular matrix components along the proximo - distal axis of the wing (Nardi, 1983). Such particles and fibres have been proven to be very important in directing cell movement and mediating adhesiveness in vertebrate neural development (Carbonetto et al., 1982). In the moth wing there is a higher concentration of these components proximally, which is the region of greatest adhesiveness for neuronal growth cones.

The nervous system of Artemia

Leydig (1851) first described the nervous system of Artemia, but this and other early papers dealt only with the general form and arrangement of the ganglia and did not include any cellular detail. Hilton (1917) summarised the information available on the central nervous system (C.N.S.) of some simple Crustacea including Artemia. He supplemented it with some details about nerve cell body form and location but did not describe the abdominal nervous system. Warren (1930) produced a simple reconstruction of an adult male Artemia nervous system from serial wax sections (fig. 1.1). He mentioned two small nerves that pass posteriorly into the abdomen from the genital ganglia but could not follow their route. A recent study by Benesch (1969) of the development of Artemia gives a detailed description of the formation of the cerebral ganglia and provides a reconstruction of the cerebral and first thoracic ganglia (fig. 1.2) with their commissures and nerves. The axons which pioneer the longitudinal connectives, transverse commissures and dorsal nerves are described in this thesis.

As well as such descriptions of the C.N.S., there have been a number of studies of sense organs and receptors of Artemia. Anadon and Anadon (1980) described the three ocelli of the adult nauplius eye, and Nassel et al. (1978) investigated neuronal types and organisation in the adult compound eye. Tyson and Sullivan described the antennular sensilla (1979), the setae of the adult trunk segments (1980), and the molar surfaces of the mandibles (1981) in adult Artemia. They identified probable mechanosensory and chemosensory cuticular receptors using scanning electron microscopy. Other mechanoreceptors have been investigated by Wolfe (1980) in his description of the frontal knob of the male-clasper. He concluded that these receptors are probably concerned with copulatory behaviour.

There have been no detailed studies of early axonogenesis in Artemia. However, some individual neurons with acetylcholinesterase (AChE) activity have been revealed histochemically in the developing nervous system of Artemia by Raineri and Falugi (1983). They propose that the first neuroblasts of the nervous system are distinguished from other neuroblasts by their precocious and stronger AChE activity. Furthermore, they describe axon bundles wrapped by AChE-positive glial cells which go from each antennal ganglion into the corresponding antennal bud, running between parallel rows of closely-packed myoblasts with AChE activity. The authors suggest that AChE may be considered as a marker for early differentiation in the nervous and muscular system of Artemia.

I have studied the developing thoracic nervous sytem of Artemia using light and electron microscopy. This investigation has revealed the presence of pioneer neurons and has also demonstrated the importance of the developing musculature of the segment in the guidance of their axons. Possible guidepost cells have also been tentatively identified.

CHAPTER TWO

LIFE HISTORY AND GENERAL BIOLOGY OF ARTEMIA

Introduction

Artemia salina (the brine shrimp) is a branchiopod crustacean from the order Anostraca. This order of simple crustaceans is also known as the Phyllopoda, in reference to the eleven pairs of thoracic appendages (phyllopods) which are borne by the adults. The brine shrimp has a world-wide distribution in salt lakes and brine ponds. In the context of this thesis Artemia salina will be referred to as Artemia.

Artemia is of major commercial importance as a food source in the mariculture of larval crustaceans and fish. A central reason for this popularity is the ease with which a population of Artemia larvae may be produced; by hydration of a chosen number of cysts, each containing a dormant Artemia gastrula. These dessication-resistant cysts are produced naturally by Artemia populations at certain times of the year in response to impending drought. Upon immersion in salt water the cysts hydrate, development of the embryo continues and within approximately 24 hours a free-swimming larva is produced. This ease of culture and economic significance of Artemia are factors that have contributed to its choice as an experimental animal in many diverse biological disciplines. These include osmoregulatory physiology, radiobiology, toxicity testing and enzyme

biochemistry. Artemia is of particular interest in developmental biology due to its phylogenetically primitive position among the Crustacea.

The earliest written record of the brine shrimp dates back to 1756 when Schlosser produced some detailed drawings of Artemia. From the middle of the nineteenth century many studies were produced concerning the morphology and taxonomy of this crustacean (eg. Leydig, 1851; Claus, 1873; Packard, 1883), but not until the early part of this century were there any studies concerning the ontogeny of Artemia. Heath (1924) described the external development of two phyllopodan crustaceans (Artemia and Branchinecta), including measurements and drawings of most stages. A later study by Weisz (1947) includes a description of the internal structure and organs of Artemia. Neither of these papers however, supplies accurate information concerning the timing and morphology of each instar. Such evidence was somewhat conflicting until Andersons' (1967) detailed study of the larval development of Artemia and another phyllopodan crustacean. This paper was supplemented shortly afterwards by the definitive overall study of Artemia developmental anatomy by Benesch (1969), who described development from the diapausing egg to the adult. That work contains very little detail concerning the development of the nervous system and the phyllopods. Olson (1979) determined the timing of developmental events between hydration of Artemia cysts and the hatching of larvae but did not investigate later instars. Growth allometry was studied by Blake (1979), whose paper includes useful measurements of width and length of the body, antennae and antennules for the first eight instars of larval development.

The following account of the life-history and external development of Artemia provides a modern account of the larval stages. This is necessary before development of the thoracic segments and the nervous system can be considered.

Life-History of Artemia

Artemia can produce both live young and dormant cysts, but for laboratory use the dessication-resistant cysts are most commonly used. Upon immersion in salt water the cysts hydrate, become spherical and development of the gastrula continues. A few hours later the cyst cracks open and the embryo at the pre-nauplius E1 stage is visible through this opening in the cyst. As embryonic development continues, the growing larva is released from the ruptured shell but is still surrounded by the hatching membrane. Larvae in this condition are at the pre-nauplius E2 stage. The first grossly visible muscular contractions occur in the antennae during the later part of the E2 stage. These contractions are very sporadic and their onset is difficult to establish precisely. However, hatching from the E2 stage to release the first instar larva is associated with a great deal of movement by the larval antennae and is completed within a minute. Approximately 24 hours after immersion the hatching membrane is ruptured to release the free-swimming nauplius larva.

First Instar

The first instar larva is orange due to the presence of yolk and bears three pairs of cephalic appendages (fig. 2.1). The most anteriorly placed of these are the small, unsegmented, sensory antennules which are tipped with two setae and one setal rudiment. These setae are bristle-like structures that also develop on other appendages of Artemia. Posterior to the antennules are the larger, biramous antennae which consist of a protopod, an endopod and an exopod. The protopod bears a basal enditic process and a distal curved seta and the endopod bears three terminal setae. The exopod bears three terminal setae (one of which is rudimentary) and seven proximal setae. These exopod setae are the 'swimming setae' which are important in the locomotory function of the antennae of the nauplius larva. The short uniramous mandibles have rudimentary setation, with two pairs of proximal setae and three terminal setae. This instar terminates with the first larval moult.

Second Instar

The second instar larva (fig. 2.2) emerges from the first moult with characteristic changes in the setation of cephalic appendages which distinguish it from the first instar larva. The proximal, non-swimming setae of the antennae and the proximal setae of the mandibles are all clearly setulated (setules are the fine, hair-like processes that develop on some setae). The antennal swimming setae are longer and hinged and the post-mandibular region is almost doubled in length. Just before the second

moult three slight segmental bulges become apparent in this region and the nauplius eye becomes conspicuously pigmented. At the posterior of the larva a slight indentation appears which is the first indication of the formation of the caudal furca.

Third Instar

The post-mandibular region of the newly-hatched third instar larva appears almost identical to that of the late second instar larva, but once again the instars may be distinguished by development of the nauplius region (antennae and antennules) (fig. 2.3). The antennae are further setulated and the basal enditic process of the protopod has bifurcated. The mandibles are unchanged and feeding begins during this instar as the embryonic yolk reserves have been exhausted. As this larval instar progresses, three further thoracic segments become externally visible in the post-mandibular region of the larva.

Fourth Instar

The larva that emerges from the third moult differs only very slightly in its nauplius region from the third instar larva but there are conspicuous changes in the post-mandibular region (fig. 2.4). The maxillae and maxillules are externally evident as paired ventrolateral swellings and the first three trunk segments bear the beginnings of evaginating phyllopods. The most anterior of these is the most developmentally advanced. This instar is equivalent to that described as the second instar by Heath (1924);

he failed to observe the second and third instar larvae later described by Anderson (1967), from whose account this description of the first four larval stages is largely based.

Fifth Instar to Ninth Instar

The larval instars that intervene between the fourth instar and metamorphosis from larva to adult at the ninth moult, are characterised by the extent of development of the post-mandibular region. This has been diagramatically summarised showing the appearance and development of thoracic segments occurring in an antero-posterior sequence (fig. 2.5). A strict sequence of thoracic segments progressively appearing and developing singly does not occur, in fact small groups of phyllopod buds form and mature together. For example, segments T1 to T3 take nine moults after initial mesoderm band segregation before their phyllopods have fully developed. However, segments T4 to T6 segregate in the mesoderm two moults later but mature at the same time as segments T1 to T3. Development of segments T4 to T6 is thus faster that the first three, which results in the first six thoracic limbs gaining maturity simultaneously, after the ninth moult. This maturity of the first six phyllopods is one of the morphological changes associated with the metamorphosis of Artemia at the ninth moult.

Tenth Instar

At metamorphosis, the locomotory and feeding functions of the antennae and mandibles are lost, these functions being assumed by the phyllopods on segments T1 to T6. The antennae of the tenth instar larvae of both sexes have rotated from a position at right angles to the body to lie in the sagittal plane (fig. 2.6a). The basal enditic process and distal curved seta of the antennal protopod have virtually disappeared to leave a small shrunken papilla. The endopod has reduced in size and in the male it flattens slightly. The mandibular palp in both sexes has lost its setae and has reduced in size, it now has a purely masticatory function. Later instars can be distinguished by their antennal features.

Further development of the antenna in the male

The morphogenesis of the male antennae is now described up to the 14th instar. In the female the antennae only gradually increase in size as does the rest of the body during these instars. Females at these stages are difficult to allocate to particular instars.

Eleventh Instar

In the male the antenna has increased in size and the axis of the exopod forms an obtuse angle with the protopod (fig. 2.6b). So that the tips of the antennae are close to the midline.

Twelfth Instar

The antenna in the male is now larger, the joint between the protopod and exopod is more distinct and the tips of the antennae cross at the midline (fig. 2.6c). There is a prominent knob on the anteromedial edge of the protopod which has been termed the 'frontal knob' (Wolfe 1980).

Thirteenth Instar

The antenna in the male is larger and bears a flattened exopod bent at right angles to the protopod (fig. 2.6d). The external margin of the antenna is developing a curved border.

Fourteenth Instar

The male antenna is still continuing to grow during this instar. The angle formed by the protopod and exopod has decreased and the two exopods overlap (fig. 2.6e). The male is sexually mature in this instar but the females are smaller and are not yet sexually mature.

Further Instars

The female has to moult once more before reproduction can take place (Anderson, 1967). Heath (1924) mentions at least two more moults after the fourteenth instar but does not describe them, Anderson (1967) counted nineteen moults in total.

Detailed information about the development of the phyllopods after metamorphosis is not available. A brief description is given by Anderson (1967) who notes that the seventh and succeeding trunk segments are added in groups as before, with the full complement of nineteen post-mandibular segments being attained at the nineteenth moult. The six abdominal segments are the last to be formed, they bear pairs of sensory setae dorsally and ventrally but no appendages. The larva will have grown from less than 1 mm long at hatching to between 8 and 10 mm long in the final instar.

CHAPTER THREE

MATERIALS AND METHODS

Maintenance of Artemia Stock

Artemia eggs ('Artemia Revolution': New Technology Ltd. Hadlow, Kent), were immersed at room temperature in filtered seawater through which air was constantly bubbled. After approximately 24 hours, larvae emerge. They are orange due to the presence of the yolk upon which they subsist for the next two days. Larvae older than two days post-hatching and also adults were fed with a suspension of yeast (DCL Active dried yeast: The Distillers Company) which was formed by mixing the dried pellets with distilled water. This suspension was added to the Artemia culture until it was cloudy. Freshly made yeast suspension was added every 2-3 days when the culture medium was almost clear. The larvae reach maturity about 3 weeks after hatching. By adding eggs to the culture at regular intervals, a population of Artemia larvae composed of many different instars was obtained. The culture tank of Artemia larvae was kept either in a daylit laboratory or in a warm room (12h at 80°F and 12h at 90°F) on a light/dark cycle of 12h L: 12h D. Other workers in this laboratory have recently found a feeding material which is preferable to yeast suspension for maintenance of Artemia cultures. It is dried phytoplankton which is obtainable from most pet shops. With this food there is less likelihood of death from disease and less clogging of the Artemia phyllopods with debris.

Normarski interference light microscopy

Artemia larvae were lightly anaesthetised in a solution containing two drops of chloroform in 10ml filtered seawater. After their appendages had stopped moving the larvae were transferred onto extra-thin (0.8/1.0mm thickness, Chance Propper Ltd.) glass slides in a drop of freshly-filtered seawater. Pieces of number 1 coverslip were placed either side of the drop as supports and a number 0 coverslip was placed over the specimen. Sufficient seawater was removed with a piece of filter-paper to ensure that the larva was flattened but not damaged. The larvae were examined using Nomarski interference optics and photographed on a Zeiss Photomicroscope II.

Fixation, embedding and Light microscopy of semithin sections

Artemia larvae were immersed in a mixture of glutaraldehyde and paraformaldehyde (Karnovsky, 1965) in a 0.1M phosphate buffer at pH 7.4 The larvae were left in this fixative for 3h at room temperature and were subsequently washed in 0.1M phosphate buffer for 20-30 minutes. This was followed by post-fixation in phosphate-buffered 1% osmium tetroxide at $4^{o}C$ for 2h. The larvae were then rinsed thoroughly in 0.1M phosphate buffer and dehydrated through an acetone series. They were then transferred to 100% propylene oxide for $\frac{1}{2}$h and finally left overnight under vacuum in 50:50 propylene oxide/Araldite. The larvae were then infiltrated with 100% Araldite over 3 days, changing the resin daily. Polymerisation was carried out at $70^{o}C$ for 3 days.

Araldite resin was made up with the following proportions of components:

Epoxy resin (Araldite CY 212)	50 ml
DDSA (Dodecenyl succinic anhydride)	50 ml
Dibutyl Phthalate	1.2 ml
DMP 30 (2,4,6-tri-dimethyl-aminomethyl phenol)	2 ml

The Araldite-embedded larvae were sectioned on a Huxley Ultramicrotome using glass knives. Serial 1µm sections were mounted in order on subbed slides, by placing them in water droplets arranged in rows on the slide. Slides were then placed on a hotplate to evaporate the water droplets. Slides were subbed (to increase adhesiveness for sections) using the following procedure.

Cleaned slides were dipped in a filtered solution of 0.1% gelatin and 0.01% chrome alum ($CrK(SO_4)_2$), and then left to dry.

The sections were stained with filtered 1% toluidine blue in a 1% borax solution and a day later covered with number 0 coverslips using Araldite as a mounting medium. The delay between staining the sections and mounting the coverslips was to avoid the stain being leached from the sections by the mounting medium.

Fixation and preparation of material for transmission electron microscopy

Larvae for transmission electron microscopy (T.E.M.) were fixed, post-fixed and embedded as described for 1µm semithin sections. Ultrathin sections were obtained using a diamond knife and a Huxley Ultramicrotome. 80-120nm thick serial sections (silver or gold coloured) were collected and picked up on uncoated 200 mesh hexagonal copper grids or on collodion films (Sjöstrand, 1967) (see fig. 3.1). The films were prepared from a 0.5% solution of collodion in amyl acetate into which were dipped cleaned, glass slides. The slides were removed from the solution and propped against an inverted beaker standing on filter paper, covered with a larger beaker (fig. 3.1a). When fully dried each slide was scored with a mounted diamond into approximately 7mm square regions. The scored side was breathed on and with this side uppermost the slide was gradually immersed, beginning at one end, into a bath of clean distilled water, causing the squares of collodion film to float off (fig. 3.1b). Each square was collected onto a steel ring (internal diameter 5mm) attached to a short steel rod (fig. 3.1c). A row of 4-8 sections was picked up on each film, according to the size of the block face, trying to place it as near to the centre of the ring as possible (fig. 3.1e). For transferring the film with sections onto a slot grid, the rod of the steel ring was inserted into a Prior micromanipulator. The slot grid was placed on a specially constructed turret and the ring lowered over the turret, orientating the sections over the slot of the grid (fig. 3.1f). The grid was pre-treated by dipping in 0.5% Formvar in ethylene dichloride to increase its adhesiveness for the collodion film. Grids of sections were stained for 20-30 minutes in

a saturated solution of uranyl acetate in 50% ethanol and then rinsed thoroughly in 50% ethanol and left to dry. They were subsequently stained in Reynolds (1963) lead citrate and washed in several rinses of boiled, distilled water, the first rinse containing two drops of 1N NaOH in 10ml water.

Lead citrate was prepared using the following proportions of components:

Boiled distilled water up to final volume	50 ml
Lead nitrate	1.33 g
Sodium citrate	1.76 g
1N NaOH	8 ml

The sections were examined and photographed using either an AEI 802 electron microscope or a Siemens 102 Elmiskop electron microscope.

Preparation of material for scanning electron microscopy

Larvae for scanning electron microscopy were fixed in Karnovsky's (1965) fixative for 3h and then rinsed in 0.1M phosphate buffer (containing 3 drops of sodium hypochlorite/50ml buffer for mild cleansing action). Larvae were dissected with a cactus spine mounted with Araldite onto the tip of a pasteur pipette, in order to expose the inner surfaces before coating. Larvae were then examined and photographed using 35mm Kodak Panatomic X film on either an ISI-60 scanning electron microscope, or a Cambridge Stereoscan 100 electron microscope.

Photography of light microscope material

Living larvae viewed with Nomarski optics or sections on slides were photographed on a Zeiss Photomicroscope II, using Kodak Panatomic X film. This was developed in Ilford Microphen developer and fixed in Amfix. Prints were made either on Ilfobrom paper which was developed in Bromophen and fixed in dilute Amfix or on Agfa Rapidoprint paper which was developed in an Agfa Rapidoprint automatic processor.

Tracing of axons from serial E.M. sections

The small size of the axons in Artemia makes it impossible to trace their pathways using light microscopy of semithin sections. Tracing axons through serial E.M. sections is a time-consuming process which I undertook by taking a huge number of photographs. For the initial analysis of serial sections, photographs were taken of the region of interest from sections on every fifth grid. Photographs showing details of pairs or bundles of axons were combined with lower-power photographs which demonstrated the features of the segmental environment of the axons at that level. From this initial analysis, regions of particular interest (such as branching of axons) could be localised to certain groups of sections which would then be studied in more detail. In some instances photographs were taken of every section on several grids. When following axons it was necessary to constantly re-refer to the original sections by viewing with a microscope. Drawings derived from photographs were not routinely used in the analysis of the data. Three-dimensional reconstruction of individual nerve fibres

was not attempted but axons were followed from section to section by marking with a pen on photographs.

Although this technique for tracing axons may seem laborious compared to the popular methods of visualising axons with dyes and fluorescent antibodies, it has the great advantage of revealing in detail the substrate over which the axons are growing.

CHAPTER FOUR

HISTOLOGY OF THE DEVELOPING THORACIC SEGMENTS

IN ARTEMIA

Introduction

The development of the thoracic segments after hatching in Artemia occurs in an antero-posterior sequence. At the posterior end of a newly hatched nauplius larva is the growth zone which progressively gives rise to the segment rudiments. Anderson (1967) described the growth zone as a region of dividing cells derived from paired rows of mesoteloblasts lying laterally on the epidermis, one row on each side of the midline, just in front of the telson. Cells are budded off anteriorly from division of the mesoteloblasts, to form two separate sheets of mesoderm cells on either side of the animal. Although formation of new segments occurs in phases of several segments at a time, correlated with alternating periods of development and moulting, each segment develops in the same way. Differentiation of the mesoderm begins dorsally with the formation of the tubular heart, while the ventral mesoderm is still an undifferentiated sheet. On each side of the animal ventral segmentation occurs as mesoderm cells assemble into pairs of bands at the anterior edge of the mesoderm sheet (fig. 4.1). These are the mesoderm rings or segment rudiments. In the thorax each pair of mesoderm bands with their associated ectoderm

subsequently grow and differentiates into a segment bearing a pair of phyllopods. The mesoderm forms the musculature of the segment, such as the gut and longitudinal muscles. This mesoderm differentiates dorsally to form the tubular heart.

This chapter presents the results of a study of the anatomy of the early development of the ventral thoracic structures, and an outline description of the development of the heart.

Results

Pre-stage 1: the undifferentiated mesoderm bands

When a mesoderm band is first segregated off from the growth zone, it consists of a monolayer two cells wide in the antero-posterior direction. The cells are spindle-shaped running medio-laterally and not closely packed. They lie on a thin layer of squamous epithelium (fig. 4.2a). Cell division within the mesoderm then increases the band width to three cells, but it remains a monolayer. The mesoderm is more closely packed at this stage and the cells are more cuboidal in shape (fig. 4.2b).

During the next stage differentiation of both the ventral mesoderm and ectoderm begins, I have designated this point in segmental development as stage 1.

Stage 1

The mesoderm band continues to increase in width in the antero-posterior direction during stage 1. In the posterior of the segment four mesoderm cells, two on either side of the midline lose their close association with the ectoderm and become spindle-shaped (fig. 4.3a). These four cells are found in the positions where at a later stage in development the dorsoventral muscles occur (Benesch, 1969). They are equivalent to the 'muscle pioneer' cells discovered in the grasshopper embryo by Ho et al. (1983). These muscle pioneers are large mesoderm cells which appear early in development and connect particular parts of the embryonic ectoderm at points which will later become insertion sites for muscle. Subsequently, the dorsoventral muscles develop as other mesoderm cells cluster around the elongated muscle pioneer fibres. The development of the dorsoventral muscle in the posterior of each segment provides a useful marker to delineate the segment boundaries (fig. 4.1).

In the anterior of the stage 1 segment the mesoderm band thickens to form a layer two to three cells deep and there are no muscle pioneers present (fig. 4.3b).

The ventral ectoderm has begun to differentiate and now consists of two types of cell; epidermis and neural epidermis. The neural epidermis forms in the anterior of the segment as two groups of cells which lie either side of the midline and are separated by a single row of cells running longitudinally along the midline. The cells of the neural epidermis are

enlarged and actively dividing (fig. 4.3b). A detailed description of the formation of ganglia from the neural epidermis is given later. The remaining ectoderm in the posterior of the segment forms epidermis.

Stage 2

As the growth of the segment continues, the dorsoventral muscle becomes more distinct (fig. 4.4a) and two lateral bulges are observed which are the first signs of phyllopod development (figs. 4.4a and b). This slight evagination results in the formation of a lumen within the bud (fig. 4.4c). At the anterior edge of the developing phyllopod, bordering this lumen, is a thick grouping of mesoderm cells three to four cells deep. More anteriorly in the segment there is no bulge visible and the epidermal cells are slightly less tightly packed than those of the evaginated region of the segment (fig. 4.4d). In this anterior region the cells of the neural epidermis are very enlarged and dividing At the most anterior edge of the segment there is little mesoderm, the epidermal cells are rounded in the region adjacent to the neural epidermis, and ganglion mother cells (which are formed from the division of neural epidermal cells) can be seen within the haemocoel (fig. 4.4e).

Stage 3

During this stage of development the phyllopod bud evaginates further and forms a distinct lobe projecting posteriorly from the ventral surface of the animal. The bud is composed of a closely packed mass of cells partly

separated from the ventral surface of the animal by a deep crease (fig. 4.5a). It is during this stage that sub-division of the initially smooth surface of the phyllopod bud begins, with the delineation of the first phyllopod region to become distinct; the exopodite (fig. 4.5b). The ectoderm of the phyllopod consists of columnar, tightly-packed cells, and round mesoderm cells destined to form the phyllopod musculature, are scattered within the developing phyllopod.

Later Stages

Subsequent stages of phyllopod development are best characterized by the extent of lobation and setation of the phyllopod. These features of the developing phyllopods have been studied using scanning electron microscopy and are described in chapter 7.

A summary table (fig. 4.6) of the major features of thoracic segment development, shows how differentiation in the ventral region of the segment as just described, relates to the formation of the heart dorsally. A brief description of the latter process now follows.

Formation of the Heart

In adult Artemia the heart consists of a tube formed from dorsal mesoderm, it stretches along the length of the animal, lying between the gut and the dorsal epidermis. This tubular heart is open to the perivisceral haemocoel at either end and also via segmentally arranged paired openings called ostia.

The heart is formed on a segmental basis and therefore develops progressively in an antero-posterior direction as do the segments. During larval development, differentiation of the dorsal mesoderm is the first indication of segmentation in the loosely-packed sheet of mesoderm cells that comprises the growth zone.

The first stage in heart formation is the appearance of two close groupings of five mesoderm cells on the dorso-lateral surfaces of the ectoderm. One of these cells on each side lies beneath the others. The cells extend long processes to contact the gut (fig. 4.7a & b). This is the first point at which the future separation of the dorsal cardiac cavity from the perivisceral haemocoel is indicated.

There are two of these early segmental units of the heart in the growth zone region, one behind the other. The anterior one involves more cells than the posterior one. As thoracic segment development continues, growth and maturation of the heart dorsally is paralleled by the ventral organisation of the mesoderm into bands which will grow and contribute to the phyllopods. Before stage 1 of segment development (which is when the ventral mesoderm and ectoderm begin to differentiate) the heart has formed a continuous mesodermal floor as mesoderm cells from either side have joined across the dorsal surface of the gut. This structure is termed the pericardial septum (fig. 4.8). The dorsolateral mesoderm has separated from the ectoderm to leave a haemocoelic space called the pericardial haemocoel either side of the main dorsal cardiac haemocoel. The dorsal longitudinal muscle is formed by mesoderm cells in the pericardial

haemocoel (fig. 4.8) and stretches longitudinally within this space from segment to segment.

Formation of the ganglia

The cells of the ganglia are derived from the division of neural epidermis cells that lie either side of the ventral midline in the anterior of each segment. Cells are budded off from this specialised epidermis into the haemocoel of the animal and will eventually assemble, differentiate and form a pair of ganglia left and right in each segment.

The neural epidermis expands and begins to divide during stage 1 of segmental development, which is the stage when differentiation of the ventral mesoderm begins with the appearance of muscle pioneer cells. In the posterior of the segment at this stage there are muscle pioneer cells beginning to establish the dorsoventral muscle and the ventral neural epidermis in this region is undifferentiated. However, anteriorly, the mesoderm band is growing and thickening and the epidermis either side of the midline is expanded and actively dividing to produce cells to form the ganglia. These neural epidermal cells are larger than those in the same region in the posterior of the segment. The neural ectoderm within the segment expands posteriorly as development of the segment proceeds, to occupy a larger area of ectoderm. By stage 3 there are two bands of neural epidermis three to five cells wide in the mediolateral direction lying either side of the ventral midline. In the posterior of the segment, where the dorsoventral muscle is inserted near the ventral midline, the neural

epidermis is displaced laterally. From the examination of sections from this and other stages of development, a picture emerges of the pattern of cell divisions that lead to the formation of a ganglion cell. This pattern is similar to that found in the grasshopper (Bate and Grunewald, 1981) and will be illustrated for Artemia by reference to sections through part of the neural epidermis of a stage 3 segment.

The enlarged cells of the neural epidermis have cytoplasm and nuclei that are much less dense than those of the adjoining epidermis. Immediately above these enlarged cells, often closely associated with them, are the daughter cells that have been budded off (fig. 4.9a). In contrast to the neural epidermis beneath them, the nuclei of these daughter cells often appear to have very condensed chromatin, and in some cases the cells are in the process of dividing (fig. 4.9b and c). Because the cells that they give rise to are the ganglion cells, those cells budded from the neural epidermis are called ganglion mother cells. Each ganglion mother cell appears to divide almost immediately after its separation from the neural epidermis, producing a pair of ganglion cells. These pairs of ganglion cells remain close together, at least initially, and can be seen in pairs, slightly lateral to the neural epidermis (fig. 4.9d).

Discussion

The general pattern of thoracic segment development described here from a study of early instar larvae, is consistent with earlier work (Anderson, 1967; Benesch, 1969). In the present study, the early sequence of

developmental events has been classed into stages numbered 1 to 4. The time taken for a segment to develop varies according to its position in the animal. For instance, later segments develop to maturity in fewer moults than the early segments (Anderson, 1967). The staging is necessary to quantify segment development in order to relate it specifically to the early development of the nervous system (see later chapters).

The earliest mesoderm cells that differentiate and begin to form the musculature of the segment are those that stretch between the epidermis and the gut dorsally where the heart will develop. Slightly later, pairs of cells are observed which connect the ventral and the dorsolateral epidermis. These mesoderm cells provide a framework around which other mesoderm cells assemble and ultimately differentiate into muscle. Similar cells have been observed in the grasshopper embryo by Ho et al. (1983), who named them muscle pioneers. The muscle pioneer cells which first stretch dorsoventrally in the thoracic segment of Artemia are located where a bundle of four muscles will develop in the mature segment. These were described by Benesch (1969) who named them I1, I2, I3 and I4. These muscles insert together as a compact group onto the ventral epidermis near the midline, but fan out to insert onto different regions of the dorsolateral epidermis. Defining the segment boundary requires cell lineage studies (Martinez-Arias and Lawrence, 1985). In the absence of data concerning cell lineage, the cell border has to be operationally defined in terms of suitable anatomical markers. The external cuticular constriction provides one such marker. Internally, segmentally repeated muscle insertions close to the level of the cuticular constriction can also

be used to define the positions of the segment borders. In this study, I have taken the insertions of muscles I1-I4 to be the anatomical feature which delimits the boundaries of the thoracic segments ventrally. The posterior segment boundary is defined as the posterior edge of the insertions of muscles I1 to I4. This is also obviously equivalent to the anterior segment boundary of the next posterior segment. This level in the segment is slightly posterior to the external constriction observed in the cuticle. It provides a clear, segmentally-repeated internal landmark which is likely to be close to the true segment border as would be defined by lineage studies. Each segment so bounded by muscle insertions will eventually have a pair of ganglia in the anterior region of the segment. Whether the mesoderm of each segment is all derived from one pair of mesoderm bands as they are segregated off from the growth zone is not known. In a study of the embryonic development of a cumacean crustacean by Dohle (1976), it was shown that the ectoderm of thoracic appendages was derived from two adjacent segments. Such a parasegmental arrangement (Martinez-Arias and Lawrence, 1985) may occur in Artemia. A cell lineage study of segmental development in the thorax would be required to elucidate these details.

CHAPTER FIVE

THE TERMINAL PIONEER NEURONS.

Introduction

Overall development of the segmental nervous system in the nauplius larva of Artemia occurs in an antero-posterior sequence. The cerebral and mandibular ganglia are present in the first instar larva at hatching (Benesch, 1969). All ganglia posterior to these develop sequentially in order. The first of these to form are the ganglia of the first maxillae, followed by those of the second maxillae, the eleven phyllopod-bearing thoracic segments and finally the fused ganglia of the two genital segments. The six abdominal segments are the last segments to develop and they do not contain ganglia. They are innervated by two longitudinal nerves which lead from the posterior side of the genital ganglion and connect with the tip of the abdomen.

At hatching, there is already an early neural connection formed between the first ganglia of the head and the as yet unsegmented posterior region of the embryo. This is established by two pairs of neurons whose somata are located near the posterior tip of the developing embryo in the telson (the telson is a permanently non-segmented region of the animal) on either side of the ventral midline. I have named these cells the terminal pioneer neurons. Using Nomarski optics the cell bodies and the proximal portions

of their axons are clearly visible through the semi-transparent cuticle of the early nauplius larva (fig. 5.1).

The two pairs of axons terminate anteriorly in the cerebral ganglia, and posteriorly branch towards the tip of the abdomen. I have not studied the establishment of this arrangement which takes place in the embryo.

These twin tracks laid along the length of the larva provide a pair of pathways for the fasciculation of later-differentiating axons which will form the longitudinal connectives that join the chain of thoracic ganglia. This chapter presents a description of the location and ultrastructure of the terminal pioneer neurons of a second instar larva, derived from studies using Nomarski optics with live larvae and T.E.M. sections of fixed animals.

Results

The somata of the two pairs of terminal pioneer axons in Artemia are situated posterior to the mesodermal teloblasts and the growth zone in the non-segmental telson (fig. 5.1), lying on the inner surface of the ventral ectoderm (fig. 5.2.). One cell body of each pair is positioned slightly anterior and medial to the other and has a larger maximum diameter, I have termed this cell terminal pioneer 1 (TP1.L and TP1.R). The other slightly posterior and lateral cell has a smaller diameter and is termed terminal pioneer 2 (TP2.L and TP2.R).

TP1 and TP2 are neurons which both extend a single, unbranched axon medio-anteriorly, these axons and the cell bodies of each pair of terminal pioneer neurons are closely associated with each other (fig. 5.1).

From the posterior pole of the TP1 cell body, a maximum of three separate dendrites projects towards the posterior of the larva (fig. 5.1). These dendrites then themselves branch in a variety of directions, with one main process always leading towards the posterior tip of the larva. In the second instar this process fasciculates with the axons of sensory neurons from the setae which develop at the tip of the caudal furca (fig. 5.1). In contrast to the branching dendrites found at the posterior pole of the TP1 cell, the smaller TP2 cell only possesses a single dendrite at its posterior pole. This dendrite also branches in the more posterior region of the larva but does not appear to fasciculate with the neurons of the terminal setae.

Terminal pioneer 1 cell body

The TP1 cells lie on the inner surface of the ventral epidermis, located at approximately three epidermal cells width laterally either side of the midline. The TP1 cell body lies on a very thin layer of epidermis. It is never actually part of the epidermal sheet and in contact with the cuticle (fig. 5.2).

The ultrastructure of the TP1 cell body is very characteristic, the cytoplasm contains two types of electron dense vesicles (fig. 5.2) which are similar to neurosecretory granules found in other nerve cells. There are

small granules (maximum diameter 1.54μm) which are often grouped into clusters and also larger granules (minimum diameter 2.5μm maximum diameter 6.2μm). Both types of vesicle are membrane-bound and they are often closely associated with the large amounts of endoplasmic reticulum seen in the cytoplasm (fig. 5.2). The extensive endoplasmic reticulum in these cells implies that they are very synthetically active.

Terminal pioneer 2 cell body

Slightly posterior and lateral to each TP1 cell body is the smaller TP2 cell body. Both the proximal regions of its axon and dendrite and the cell body of the TP2 cell contain the same dense accumulation of large and small membrane-bound vesicles (figs. 5.3 and 5.4) as were observed in the TP1 cells. A fine, hair-like structure of presumed sensory function projects from the cell body of the TP2 cell to the exterior of the larva. The cell body is inserted into the epidermis in the region where this hair emerges, which is anterior to the nucleus of the cell. The cell membrane at this point is ruffled and infolded (fig. 5.4). The short, hair-like structure contains a number of long microtubules that extend into the cell body towards the basal surface of the cell. A basal body-like structure can be seen within the cell near the point where the hair projects from the cell (fig. 5.5). The posterior of the cell body, where the nucleus is located, lies within the haemocoel on a thin layer of epidermis (fig. 5.6a). A deep cytoplasm-filled cleft can be seen in the nucleus in this region (fig. 5.6b).

Discussion

The early appearance, the form and the location of the cell bodies and axons of the TP neurons of Artemia strongly suggest that they belong to the class of cells known as pioneer neurons. Pioneer neurons were first described in an arthropod by Bate (1976) who studied the embryonic metathoracic leg and the antennae of the grasshopper. Since then a wide variety of central and peripheral neurons have been described in insects which establish the first neural pathways early in development (eg. Goodman et al., 1982; Bentley and Keshishian, 1982a). The TP neurons of Artemia satisfy the essential diagnostic features of pioneer neurons. Firstly, they arise at a undetermined but early stage in development and send axons over uninnervated regions of the embryo to connect parts of the animal which will later be separated due to growth and expansion. These axons span what will eventually be almost the whole length of the adult from the head to the telson. Secondly, the results presented in the next chapter show that these TP axons are followed by other axons and that they provide a crucial portion of the framework of the Artemia thoracic nervous system.

There appears to be no specific class of precursor cell which gives rise to pioneer neurons. They may for instance be derived from limb-bud ectoderm (Keshishian, 1980), from neuroblasts or midline precursors (Bate and Grunewald, 1981) or even wing sensilla (Murray et al., 1984). The exact origin of the TP neurons of Artemia is not known but they are probably sibling cells derived from the division of an ectodermal cell, with

one cell (TP2) remaining within the epidermis. The majority of peripheral pioneer neurons in insect appendages are pairs of cells that are the progeny of the division of an ectodermal cell. Unlike the TP neurons however, these insect neurons both migrate out of the epithelium and lie as a pair within the lumen of the appendage (Keshishian, 1980).

The TP neurons are bipolar cells. Two proximally unbranched axons lead from them anteriorly towards the head and these are followed by many axons of other neurons which develop later (see chapter 6). The TP axon pairs are comparable to the centrally directed axons of peripheral pioneer neurons in insects. Branching dendrites emerge from the posterior side of the TP neurons and grow towards the posterior of the larva. These dendrites may also serve a role as pioneer fibres, probably for the axons of neurons which innervate the many setae that develop at the caudal tip of the larva. Apical dendrites have been observed on pioneer neurons in the grasshopper limb-bud (Keshishian, 1980) and also the cercus of the cricket (Edwards and Chen, 1979). The structure of the dendrites in the cricket cercus suggests that these neurons may be modified sensory neurons perhaps derived from type 1 sensory neurons (Bate, 1978), commonly associated with cuticular receptors. The lack of supportive cells and the absence of any structure for sensory transduction in the insect pioneer neurons implies that they no longer serve a sensory function. In Artemia, the microtubule-filled cuticular peg of TP2 may be evidence that the TP cells are also modified sensory neurons and perhaps TP2 retains some (probably mechansensory) sensitivity. Electrophysiological studies would be required to investigate this further.

The present account is the first to identify TP1 and 2 as pioneers and to describe their anatomy in detail.

CHAPTER SIX

EARLY DEVELOPMENT OF THE VENTRAL THORACIC NERVOUS SYSTEM

Introduction

Pioneer neurons have been studied extensively in insects, but have not been investigated in detail in any other arthropod group. The embryo of the crayfish Procambarus however, has been mentioned briefly by Thomas et al., (1984), who included this decapod crustacean together with the grasshopper and Drosophila embryos into a group of embryonic arthropods all of which are very similar in terms of their identified central pioneer neurons. The majority of the insect studies have used such techniques as cobalt filling, fluorescent dye injection or monoclonal antibody labelling of neurons to visualise the axons. Such methods do not reveal any ultrastructural details about the axons or their environment, although dye-coupling between cells and variations in some cell-surface properties have been usefully determined (see Introduction).

This chapter presents the results of an electron-microscopical analysis of the location, ultrastructure, branching and fasciculation of the pioneering axons seen in the early stages of thoracic segment development in Artemia.

Results

Neural development in the growth zone and the newly-segregated mesoderm bands

The somata of the two pairs of TP neurons lie ventrally in the unsegmented telson, at the posterior end of the larva. Each neuron possesses a single, proximally unbranched axon which leads medio-anteriorly with the other axon of the pair (see chapter 5) via the growth zone region, where the thoracic segment rudiments are being formed, and thence to the head. These two pairs of axons are the only elements of the framework of the nervous system that are present at the growth zone and the newly-segregated, undifferentiated mesoderm band level.

The TP axons are situated beneath the basal lamina of the ventral epidermis and are surrounded by numerous processes from epidermal and mesodermal cells and occasional empty-looking vesicles which may be swollen cell processes (fig. 6.1). The axons do not lie on any distinctive feature of the epidermal surface, sometimes they are found at the junctions between cells or sometimes in small grooves on their basal surfaces.

Ultrastructure of the TP axons at the growth zone and newly-segregated mesoderm band levels

Several criteria are used to discriminate between the profiles of axons and the processes of surrounding ectodermal and mesodermal cells. A primary one is continuity; although the sizes and shapes of the axon profiles vary (fig. 6.1), the axons are continuous from section to section. Parts and processes of other cells soon disappear after a short distance. Axons always contain microtubules (fig. 6.2), whereas other cell processes only occasionally have them. The cytoplasm of the axons is almost always of a less dense appearance than the cytoplasm of surrounding cells. This characteristic is even more noticeable in some regions where one of the axons is swollen relative to the other (fig. 6.1). This feature, where one axon is swollen, is seen at regular intervals along the TP axons in these earliest stages of thoracic segmentation. Both axons in these specialised regions show a distinctive change in their shape, size and axoplasmic constitution. These features will now be listed:

(1) The less dense axoplasm (fig. 6.1).

(2) There are always mitochondria in both axons in this region (figs. 6.1 and 6.3), whereas mitochondria are only sparsely scattered in the cytoplasm at other levels. The mitochondria in both axons in this region are randomly orientated (fig. 6.3), while those in the rest of the axon always have their long axes orientated parallel to

that of the TP axon (fig. 6.2). Most, if not all of these mitochondria have an extension of their outer membrane which forms a vesicular protrusion attached to the mitochondrion (fig. 6.3c,d,e,f,g). This phenomenon has been observed in a wide variety of plant and animal tissues and is not considered to be an artefact (Spacek and Lieberman, 1980). In one instance this vesicular protrusion of outer mitochondrial membrane (OMM) appears to be associated with smooth endoplasmic reticulum (fig. 6.3g). This provides support for one of the theories proposed to explain these membrane extensions: that the OMM is continuous with the endoplasmic reticulum and is a part of the cell's endomembrane system. (Spăcek and Lieberman, 1980).

(3) There is often some electron-dense, granular material in the axoplasm of one of the two axons in each TP pair (figs. 6.3a,b,e,f).

(4) The two TP axons seem to have a greater affinity than usual for each other in these regions, in some instances one axon is almost totally encircled by the other axon (figs. 6.3 *c* and f). Due to this affinity of the axons for each other, often they have quite irregular profiles, which contrasts with the roughly circular profiles of the other stretches of the axons (fig. 6.1a).

There are many vesicles present throughout the axoplasm, they vary in size and resemble the vesicles formed by the extensions of the outer membrane of mitochondria (figs. 6.1a and f and 6.3).

Neural development at the Stage 1 and Pre-Stage 1 segment levels

After a mesoderm band has been segregated off from the growth zone, it grows and thickens before beginning to differentiate ventrally in stage 1 of development. Just before stage 1, two new pairs of axons appear in the developing segment. These pairs of axons are derived from the dorsal setae (see chapter 8) and grow at right angles to the TP axon pairs. They are destined to be the first of many nerve cells to join the TP axons. In the pre-stage 1 segment, the dorsal setae axons have begun their outgrowth but they have not yet reached the TP axons (fig. 6.4).

The TP axons in the mesoderm band region just before it begins to differentiate in stage 1, show none of the periodic swellings seen at the growth zone and early mesoderm band levels. The two axon profiles are approximately equally sized and circular in profile (fig. 6.5).

During stage 1 the ventral ectoderm and mesoderm begin to show signs of differentiation. Some mesoderm cells begin to assemble for muscle formation and some ectoderm becomes neural ectoderm. There are however no major changes detected in the developing nervous system. The two TP axons remain closely associated with the epidermis (fig. 6.6) and the dorsal setae axons have not yet reached them (fig. 6.4).

Neural development in the Stage 2 segment

There are two significant developments in the formation of the embryonic nervous system during stage 2 of segmental development. The pairs of axons from the dorsal setae reach and fasciculate with the terminal pioneer axons and the first axons to pioneer a transverse commissure pathway begin to grow out.

In the posterior of the segment, where the posterior edge of the dorsoventral muscle is inserted onto the epidermis, the pairs of axons from the dorsal setae join with the TP axons (see chapter 8 for detailed account). One of the two axons that grows out from each dorsal seta bifurcates when it reaches the TP axons, sending one branch anteriorly and one branch posteriorly. I have termed this axon dorsal seta 1 (DS1). The other axon of the pair, which I have termed dorsal seta 2 (DS2) does not bifurcate but turns and grows anteriorly when it reaches the longitudinal TP axons (fig. 6.4).

As a consequence of this arrangement, posterior to the junction where the dorsal seta axons reach the TP axons, there are three axons visible, two of which are the TP axons and the third is the posterior branch of DS1. (fig. 6.4 level A and fig. 6.7). However, anterior to the junction where the dorsal seta axons join the TP axons (fig. 6.4 level C), there are four axons visible in the position of the TP axons. Two of these are the TP axons, one is the anterior branch of DS1 and one is DS2 which has turned anteriorly (fig. 6.8).

This pattern of fasciculation and branching of the dorsal seta axons at the junction with the TP pair, was revealed by two sets of serial sections from different specimens through this region (fig. 6.4 level B). The transverse profiles of the TP axons are always very similar to each other and generally rounded. The profiles of the two DS axons are much more irregular in this region (figs. 6.9(a) and (b)) as they reach the TP axons from the lateral side and fasciculate with them. This fasciculation of the dorsal seta axons with the TP axons is the first evidence that the TP neurons are indeed pioneer neurons.

More anteriorly in the segment there are five axons in the longitudinal tracts, as another axon (from an unknown neuron) has joined the bundle of two TP axons and two dorsal seta axons. This fifth axon could be derived from one of the dorsal seta axons of the next anterior segment, the identity of this axon requires further experimental investigation. At the anterior edge of the dorsoventral muscle insertion (which is inserted in the posterior portion of the segment), two axon branches grow out from the pair of TP axons in the longitudinal tract. These axons are the pioneers of the posterior commissure, they grow out at a level in the segment where the anterior edge of the dorsoventral muscle meets the posterior edge of the group of newly-formed ganglion cells (fig. 6.10). These axons which pioneer the posterior commissure grow only a short distance medially in this stage (fig. 6.11).

Neural development in the Stage 3 and Stage 4 segment

Location of the commissures

It was already established in the stage 2 segment that the posterior commissure axon branches grow out from the TP axons at a position in the segment which is at the anterior edge of the dorsoventral muscle (fig. 6.10). These axon branches can still be seen at this location in the stage 3 segment (fig. 6.12 and fig. 6.13b). No other axons have fasciculated with them at this stage of development.

Further branches emerge from the TP axons in the anterior of the stage 3 segment, and grow medially to pioneer the anterior commissure pathway. They grow out in the region of the segment boundary, beneath the posterior edge of the dorsoventral muscle of the next anterior segment (fig. 6.13a). These two axons are followed in stage 3 by a pair of axons L1 and L2 (fig. 6.13a and fig. 6.14) which appear from an unknown location lateral to the TP axons and grow along them towards the midline. These first four axons in the anterior commissure grow between the cell bodies of the developing ganglion at its anterior edge. At a level just medial to the junction where the TP axons branch into the anterior commissure pathway, these axons and L1 and L2 are much smaller than at the junction and are filled with dense axoplasm including many mitochondria (fig. 6.14). L1 and L2 do not extend far across the commissure during stage 3. For most of the length of both commissure pathways during this stage, each contains only the two axon branches from the TP neurons.

The courses of these axon pairs in the two commissure pathways follow the edges of the dorsoventral muscle insertions over the surfaces of ganglion cells (fig. 6.15).

At the level of the ventral midline, there is only a single layer of epidermis. In the posterior commissure pathway near the midline there are four axons, where axon branches from the left and right sides have overlapped for a short distance (fig. 6.16). In the anterior commissure pathway at this level there are two axon branches of the TP neurons. These anterior commissure axon, near the midline have wide, thin, flat profiles in contrast to the more rounded appearance of the four axons in the posterior commissure pathway (fig. 6.17). This flattened appearance of the ends of the axon branches in the anterior commissure is similar to that described for nerve growth cones (Letourneau, 1983). This could indicate that the axons are exploring the environment and pathfinding, whereas the posterior commissure axons have overlapped with their equivalent contralateral pair and are closely following them.

Stage 3 is thus the earliest stage during which a connection is formed between the left and right halves of the thoracic nervous system. Both commissure pathways are completed in the stage 4 segment, and the branches of the TP axons have been followed by many other axons (fig. 6.16).

Discussion

The TP neurons in the thorax of Artemia establish the pathways of the longitudinal connectives which link together the chain of ganglia, and they branch to pioneer the two commissures which connect the left and right ganglia of each segment. The involvement of these neurons in the cerebral and abdominal nervous system of Artemia was not investigated.

The ultrastructure and location of the TP neuron cell bodies was discussed in chapter 4. It was shown that they lie in two pairs on the inner epidermis of the telson at approximately three epidermal cells width either side of the ventral midline. The axons, which lead longitudinally from the anterior poles of these bipolar cells and link up to the cerebral ganglia, also lie at approximately this same medio-lateral level. The ultrastructural analysis presented in this chapter showed that these pairs of axons do not appear to lie on any distinctive anatomical feature of the epidermal or mesodermal cells that they overlie. Since the original outgrowth of these axons from the TP neurons has not been studied, it is possible that their axonal growth cones were channelled by some morphological feature which is now no longer evident. The cellular substrate upon which the axons lie later in development may simply be a consequence of axonal elongation over the differentiating post-naupliar segments. The axons do apparently exhibit an affinity for the cells of the ventral surface of the larva. At any particular level one or both of the TP axons is closely apposed to the surface of underlying cells. At least in the early stages of segment development (pre-stage 1) it was also observed that the TP axons are

closely associated with the epidermal basal lamina, being sandwiched between it and the epidermis. Whether the basal lamina or the underlying cells are ever involved in the guidance of the first pioneering growth cones from the TP neurons remains to be investigated.

The significance of the periodic variations in the structure of the pairs of TP axons is not clear. There is a concentration of mitochondria in these regions, most of which have extensions of the outer mitochondrial membrane (OMM), which may be bigger than the mitochondrion itself. Similar extensions of the OMM have been observed in many vertebrate tissues and also in crayfish lateral giant fibres (Peracchia, 1973). Their occurrence may be more widespread in other tissues and species, but until recently they were probably considered artefactual. Spăcek and Lieberman (1980), have suggested that these extensions of the OMM are continuous with the smooth endoplasmic reticulum and that the mitochondria (inner membrane and matrix) are transported along the axon within this tubular network. Alternatively, the OMM extensions may result from metabolic activity of the mitochondria, perhaps these special regions of the axon are sites of energy production by groups of mitochondria. Also characteristic of these axonal regions is an accumulation of electron-dense granular material. This material may either have been transported there or could be newly-synthesised if this is indeed a site of metabolic activity. It may be significant that these characteristic features are observed where the TP axons are beginning to branch medially to pioneer the posterior commissure in the stage 2 segment. Such swellings of the TP axons in pre-stage 2 segments may represent the sites where the TP axons will branch at a

later stage of development. Periodic swellings of pioneer axons have not been reported in the insect studies, although it is not certain whether they would be revealed by the techniques commonly used.

Branches of the TP axons grow out only a short distance medially in the stage 2 segment to begin the posterior commissure pathway. They grow out at an antero-posterior level in the segment where two cell types meet. That is, where the anterior edge of the dorsoventral muscle meets a group of ganglion cells. Completion of the posterior and anterior pioneer commissure pathways by TP axon branches occurs during stage 3. Both pairs of commissure axons grow over ganglion cells for most of the distance to the midline. As they approach the midline they grow over a monolayer of epidermal cells. Both pairs of axons are also situated ventrally below an edge of the dorsoventral muscle, either in contact with the muscle or with mesoderm cells between the axons and muscle. This dorsoventral muscle is inserted in the posterior of the segment in the region of the ventral midline and occupies a wide band in the antero-posterior axis of the segment. In the stage 3 segment for example, the dorsoventral muscle at the midline is inserted onto nine of the thirteen epidermal cells that constitute the length of the segment. The axon branches that pioneer the posterior commissure grow out at the level of the anterior edge of the dorsoventral muscle of the same segment. The axon branches that pioneer the anterior commissure grow out at the level of the anterior border of the segment. This is at the posterior edge of the dorsoventral muscle insertion of the adjoining next anterior segment. Sometimes the axon branches are actually in contact with the muscle

fibres but often they are sandwiched between ganglion cells which lie directly beneath the ventral surface of the edge of muscle. It may be that the muscle, ganglion and epidermal cells between or over which the axons grow, have a special affinity for the pioneer axons and that they guide them medially towards the ventral midline. From observation of the initial emergence of the posterior commissure axon branches during stage 2, it appears that the pioneer fibres grow out medially and never laterally. It is unknown what determines the correct polarity of their outgrowth, although a cell adjacent to the TP axons in this region may act as a 'guidepost' cell for the axon branches as has been demonstrated for axons pioneering the commissures in the grasshopper (Goodman et al., 1982).

The ways in which this similar ladder-like framework of longitudinal connectives and transverse commissures is constructed by pioneers neurons in the grasshopper differs considerably from the pattern described here for Artemia. There are no cells in the grasshopper embryo that are equivalent in function to the TP neurons. The first pathways of the thoracic connectives and commissures in this insect are established on a segmental basis by several pioneer neurons which are located both centrally and peripherally (Bate and Grunewald, 1981; Goodman et al., 1982). The axons of these neurons grow over the basal lamina and contact other neurons (guidepost cells) along their route. For most of their path they do not grow over cell bodies. Axons growing longitudinally eventually meet and fasciculate with their homologues from neighbouring segments, and those growing medially also meet and fasciculate with their contralateral homologues, as was observed for Artemia. So where Artemia uses only

two neurons to form each half of the ladder of the nervous system for the whole thorax, the grasshopper needs 8 neurons to pioneer half of the ladder in each thoracic segment (fig. 6.18). Further studies of the later development of the embryonic grasshopper have revealed that each connective or commissure is composed of up to 20 separate fascicles and that in each commissure each of them is separately pioneered by a different neuron (s) (Bert Stewart, pers. comm.). Further studies of later developmental stages are required to see if a similar arrangement occurs in Artemia.

CHAPTER 7

DEVELOPMENT OF THE EXTERNAL MORPHOLOGY

OF THORACIC SEGMENTS AND PHYLLOPODS

Introduction

The significant internal structural features of early thoracic segment development have been described in chapter 4 and related to the development of the nervous system in chapters 5 and 6. This chapter presents the external features of thoracic segment development in order that the morphogenesis and setation of the phyllopods may be correlated with the early formation of the nervous system. This study also provides further information for the consideration of the significance of Artemia in crustacean phylogeny.

It was shown in chapter 4 that the first internal indication of thoracic segmentation occurs dorsally, with the assembly of mesoderm cells from an undifferentiated sheet into an arrangement of cells destined to form a segmental part of the heart. The first external indication of thoracic segmentation is also dorsal, with the appearance of a pair of setae either side of the dorsal midline before stage 1 (fig. 7.1) (see chapter 8 for detailed account). There are no similar markers on the ventral part of the segment, from which the phyllopods will emerge, until stage 1 of

development. This is the stage when ventral differentiation of the mesoderm begins, with the appearance of the muscle pioneer cells which form the basis of the dorsoventral muscles. Externally at stage 1 there is a slight circumferential groove in the epidermis and cuticle of the segment (fig. 7.2). During the subsequent stage 2 the phyllopod rudiments emerge as slight bulges projecting from the ventro-lateral surface of the larval segment. In stage 3 the first infoldings appear in the surface of the phyllopod bud and during stage 4 the first seta of the phyllopod appears. Subsequently, the different regions of the phyllopod differentiate and grow. The adult phyllopod, composed of flattened lobes, many bearing setae, gradually differentiates. This developmental sequence is summarised diagrammatically in figure 7.3.

Although descriptions of only the first stages of thoracic segment development were needed for correlation with early neuronal development, this study was extended to provide a full account of the process of phyllopod differentiation.

Results

Stages 1-3

During the first two stages of thoracic segment development the phyllopod is visible as a smooth bulge, but during stage 3 the first crease forms in its surface (figs. 7.2 and 7.3). This first infolding occurs between the regions that will become the exopodite and the endopodite. The exopodite

region is clearly delineated in the next stage (4) and it is thus the first discrete lobe of the Artemia phyllopod to appear. The exopodite in the mature phyllopod is the most distal lobe. The lobes that are formed proximal to it on the outer side of the limb are termed exites and those on the inner side are called endites (Cannon, 1926).

At the beginning of stage 1 the nervous system of the thoracic segment consists only of the two pairs of terminal pioneer axons and the pairs of neurons growing down from the dorsal setae towards the ventral surface. But during stages 2 and 3, when the phyllopod begins to evaginate and forms the first fold in its surface, these earliest axons in the thoracic segment begin to link up. In stage 2 the axons from the dorsal setae which are located in the posterior half of the segment fasciculate with the terminal pioneer axons and the pairs of axons that pioneer the posterior commissure begin to grow out. By the end of stage 3 the longitudinal tracts formed by the terminal pioneers contain several axons and are connected across the ventral midline by the axon branches that pioneer both commissures.

Stage 4

During this stage the exopodite is visible as a discrete region on the phyllopod bud (figs. 7.2 and 7.3). It is during this stage that the setation of the phyllopod begins. The first seta (S1) develops during stage 4 on the exopodite as a smooth protruding bulge of tissue (fig. 7.4). Three more infoldings form on the endite (inner) surface of the bud, these folds lie

lie between the regions that will form the four endites. That is between endites 3 and 2, 2 and 1 and endite 1 and the proximal endite (fig. 7.5). The endopodite is not delineated from endite 3 during this stage. The exite surface of the stage 4 phyllopod also shows further differentiation with the appearance of a fold between the bract (an exite) and the proximal exite (fig. 7.1). To summarise the developmental events of this stage : the first discrete lobe of the phyllopod is clearly distinguishable and bears the rudiment of the first seta. Subdivision of other future regions of the phyllopod is indicated by the formation of creases between them, except endite 3 and the endopodite (fig. 7.3).

The nervous system is well-advanced in stage 4. There are many axons in the connectives and commissures, and the pair of ganglia contain many cell bodies.

Stage 5

During this and later stages there is a gradation of development from the early to the late part of the stage which is quantifiable according to numbers of seta rudiments on different lobes of the phyllopod (fig. 7.3).

Proximal exite and bract

The proximal exite and bract are rounded lobes (fig. 7.2), the surface of the bract bears a region which is highly folded (fig. 7.4). The proximal exite bears no folded area, it grows into a shorter but wider lobe than the

bract in later stages (fig. 7.2). Neither of these two exites ever bears setae.

Expodite and endopodite

The first seta (S1) which appears in a rudimentary form on the exopodite in stage 4 is more distinct in this stage (fig. 7.4). Early in stage 5 the last major subdivision is formed; a crease develops between the endite 3 and endopodite regions (fig. 7.5). All the other slight infoldings that were formed during stage 4 are now very distinct (fig. 7.3).

Endites

In the early part of stage 5 there are further setae which appear on endites 3 and 2. On endite 3 there are two seta rudiments, one of which (S2) is larger than the other (S3) (fig. 7.6), and on endite 2; two seta rudiments are seen (S4 and S5) (fig. 7.6). Later in stage 5 there are more setae present, two develop on endite 1 (S6 and S7) and a single seta on the proximal endite (S8). During this stage the endites show the first indication of the organisation of the setae into two rows along the margins of the lobes: the anterior edge row and the posterior edge row (fig. 7.3). S2 to S8 all project from the anterior edges of the endites. The first posterior edge setae begin to appear on the endites late in stage 5 (fig. 7.7; right side). One of the distinguishing features between stage 5 and 6 is that all the setae in stage 5 remain small and rudimentary.

Stage 6

The different lobes of the phyllopod during stage 5 are of similar proportions to each other and are each of a rounded uniform shape. During stage 6 these different lobes each begin to acquire their characteristic morphology (fig. 7.3).

Proximal exite and bract

The proximal exite and the bract begin to differ from each other during this stage as the bract expands distally and the proximal exite remains rounded (fig. 7.2).

Expodite and endopodite

Early in stage 6 the exopodite bears two setae; S1 that first appeared in stage 4, and a new shorter one (S9) next to it on the medial side of the exopodite (fig. 7.4). During stage 6 another seta (S10) develops on the exopodite on the lateral side (fig. 7.7; left and right sides).

The segregation of the endopodite from endite 3 is already completed in stage 5, and during stage 6 the endopodite subdivides into three sections : A, B and C (figs. 7.3 and 7.6). Early in the stage, section C, which is next to the exopodite, bears a single seta (S11) (fig. 7.6) and sections A and B are delineated by slight folds. As the stage progresses two seta rudiments (S12 and S13) appear on section A (fig. 7.7; left side). The

relative sizes and shapes of the three sections of the endopodite change during stage 6, section C expands greatly relative to the other two sections (fig. 7.7; right side).

Endites

An accurate description of the setation of the anterior edges of the endites in stage 6 is possible, but the numbers of setae on the posterior edges is variable. S2, one of the two setae on the anterior edge of endite 3 is slightly longer than S3 at the beginning of the stage and towards the end of the stage is the longest of the anterior edge endite setae (fig. 7.6). S4 and S5 on endite 2 are always approximately equally sized and S6 on endite 1 is a similar size to these two (fig. 7.6). There is only ever a single centrally placed seta (S8) on the anterior edge of the proximal endite. The numbers of the anterior edge endite setae do not change after stage 6 (fig. 7.3).

On the posterior edge of endite 3 there are two (fig. 7.6) or three (fig. 7.7; left side) setae, they are smaller than the anterior ones. The posterior edge of endite 2 bears up to six setae (fig. 7.7; left side) and that of endite 1 bears between three (fig. 7.6) and eight (fig. 7.7; left side) setae. Posterior to S8 on the posterior edge of the proximal endite there are up to twelve setae (fig. 7.7).

Stage 7

Stage 7 is the stage in which the last major morphological changes occur in the developing phyllopod. Further development of the phyllopod after stage 7 involves a change in size of the whole appendage and an increase in the length and number of setae but the basic structure is that which is finally established in stage 7 (fig. 7.3).

Proximal exite and bract

The different shapes of the proximal exite and bract are clear in this stage as the bract continues to develop into a long lobe compared to the shorter, rounded proximal exite (fig. 7.2).

Expodite and endopodite

The exopodite in stage 7 bears three (S9, S1, S10) (fig. 7.7; right side) or four (fig. 7.8) setae. The expansion of section C of the endopodite relative to the other sections continues in this stage and it becomes the widest of all the phyllopod lobes (fig. 7.8). Section C bears up to four setae in this stage (fig. 7.8), section B is clearly defined and section A still bears two setae (S12, S13) on its anterior edge. The further development of section C of the endopodite is similar to that just described for the exopodite, that is the gradual addition of more setae on either side of the original seta (S11) (figs. 7.7 and 7.8). Section B of the endopodite remains small. The unusually long S2 on endite 3 continues to

grow in length relative to other endite setae during this stage, and this difference becomes accentuated as the phyllopod increases in size until S2 becomes the longest endite seta (fig. 7.9).

Endites

The number of setae on the anterior edges of the endites remains the same as that established during stage 6, and estimation of the numbers of setae on the posterior edges of the endites is difficult. This is because they are often obscured by those on the anterior edges and because they form a continuous row along the endites and cannot always be definitively allocated to a particular endite. The maximum number for each endite does not appear to vary significantly from the numbers in stage 6 and progressively increases towards the ventral midline from two on endite 3 to approximately twelve on the proximal endite (fig. 7.10). The relative size of the endites also increases towards the ventral midline, the proximal endite therefore being the widest (fig. 7.10). The length of the endite setae on the posterior edges in this stage is such that they can meet those of the opposing limb along the ventral midline (fig. 7.8).

Seven pairs of phyllopods reach this stage of development before metamorphosis from larva to adult occurs at the ninth moult (fig. 7.9).

Phyllopod development after metamorphosis

At metamorphosis the nauplius second antennae lose the locomotory and feeding functions which they fulfilled for the larva and they gradually become sensory appendages in the female and graspers in the male (see Chapter 3).

At the ninth moult the shrunken second antennae rotate from being in the horizontal plane where they point laterally (fig. 7.9), to lie in the dorsoventral plane, projecting ventrally. At the same time as the antennae rotate, the phyllopods change their alignment in a similar fashion, moving medially towards each other. The row of endites on the phyllopod comes to lie almost in the transverse dorsoventral plane and faces the contralateral row across the ventral midline (fig. 7.11). The overlapping endopodites form the most ventral aspect of the phyllopods and the overlapping exopodites are the most lateral and distal lobes. In this orientation the phyllopods form an effective filter-feeding mechanism where water and food are drawn into spaces between phyllopods, these spaces being bounded by the carefully arranged lobes of neighboring phyllopods (Cannon, 1933). This orientation of phyllopods also forms a distinct channel, the food groove, along the ventral midline between the left and right rows of phyllopods (fig. 7.11).

Discussion

The phyllopods of Artemia develop gradually over several instars from smooth bulges into flattened, leaf-like appendages composed of lobes. This morphogenetic process is at a very early stage when the framework of the segmental nervous system has been completed by pioneer neurons. Sub-division of the phyllopod bud into lobes begins in stage 3, by which time the pathways of the longitudinal connectives and transverse commissures have been formed in the segment by the TP neurons. In the stage 4 segment the nervous system is well developed, with many axons in the commissures and connectives and many cells in the developing pair of ganglia. Phyllopod sub-division however is still only at an early stage in stage 4 with the complete delineation of the first lobe to form (the exopodite) and the appearance of the first rudimentary seta on this lobe. The formation of the central nervous system is thus much advanced at a very early point in phyllopod morphogenesis. It is not known at what stage the first axons connect the phyllopod with the C.N.S. Pioneer neurons may arise within the phyllopod bud during stages 1-4 which is before distal expansion of the appendage and therefore when distances are short, as has been observed in insect appendages (Bentley and Keshishian, 1982b). The two axons L1 and L2 that are first found on the anterior commissure pathway during stage 3 (see chapter 6) come from an unknown lateral location which could be the phyllopod bud. This possibility needs further investigation but could indicate that major central (connectives and commissures) and peripheral (dorsal nerve and phyllopod nerve) pathways are all completed and interconnected in stages 2 and 3. If one (or more)

nerve pathways are pioneered between the phyllopod bud and the C.N.S. at this early stage in development, these routes would then be available for the guidance of axons from the neurons which innervate the many setae that develop in the next stages. Benesch (1969) described a total of three nerves that lead from each side of the thoracic ganglia into the phyllopods.

The study presented in this chapter is the only detailed analysis available of the growth and differentiation of a branchiopod thoracic appendage. From observations of the appendages of adults and some larval stages of other Crustacea, a description has emerged of the basic structure of a crustacean appendage (McLaughlin, 1982). Although the detailed structure of the lobed Artemia phyllopod appears to be quite different to the many-segmented appendages of higher Crustacea, its general structure does fit the basic pattern. With the exception of the antennules, all crustacean appendages are essentially biramous, they bear an external exopod and internal endopod on a basal protopod. There are also lateral protrusions from the protopod which are the exites and medial protrusions which are the endites. The first morphogenetic event in the differentiation of the smooth surface of the Artemia phyllopod bud is the appearance of a crease between the regions that will form the two terminal lobes; the expodite and the endopodite. This crease therefore represents the first indication of the bifurcation of the phyllopod bud to form a biramous appendage. As well as being the first discreet lobe to form, the exopodite is also the first lobe to develop a seta. The endopodite, endites and exites develop subsequently and thus the phyllopod differentiates in a broadly disto-

proximal manner. Developmental information of this sort can be usefully combined with the extensive descriptions of exoskeletal features of adults and some larval stages that have always played a major role in the discussion of crustacean evolution (Whittington and Rolfe, 1963). Much interest has been focused in these discussions on presumed primitive groups such as the Branchiopoda and, more recently, the Cephalocarida. The latter are a newly-discovered subclass considered to be more phylogenetically primitive than the former (Sanders, 1957). Many authors have constructed evolutionary schemes based on purely anatomical features, devising theoretical correlations between the different parts of crustacean appendages. These schemes have included branchiopods, cephalocarids and other extant species, as well as information from fossil species such as Lipostraca (Cannon, 1933) and Lepidocaris and Neolonus (a trilobite) (Sanders, 1957). However, there are many problems inherent in the consideration of evolutionary links and the designation of primitive features when derived from anatomy alone. Convergent evolution rather than relatedness could be responsible for many common features, and structural simplicity can originate secondarily in more advanced Crustacea, as well as being indicative of true primitiveness. Developmental studies can help to resolve these possible ambiguities and provide a more complete picture of the origin of particular parts of crustacean appendages. These studies reveal the occurrence of fusion or expansion of regions which would otherwise be unobserved. The mature Artemia phyllopod for instance, possesses six endites in a row, whose setae merge into a continuous row for filter-feeding. However, only the proximal four of these are true endites that originate as protrusions from the protopod. The two distal

endites are formed when the endopodite subdivides into three sections early in its development. Comparably detailed studies of appendage morphogenesis are not yet available for Crustacea from other groups that are proposed to be closely related to Artemia in evolutionary terms.

From the observation of one specimen whose left and right sides were out of synchrony with each other by nearly a whole stage, it appears that the phyllopods do not necessarily develop as a segmental pair, but rather that the left or right row of phyllopods develop as a group. Comparable phenomena have been observed in both natural and x-irradiated populations of Artemia. Mosaic individuals have been described, such as perfectly bilateral gynandromorphs or animals where five appendages on the left thorax are mixtures of male and female genitalia (Bowen et al., 1966). As well as phyllopods developing as mixtures of genitalia other mutant Artemia have been described whose external genitalia on both sides are composed of a mixture of ovisac, penis and phyllopod structures. Furthermore, by varying the concentrations of purines, pyrimidines and amino-acids in the nutritive medium, it is possible to induce supernumerary genitalia on all abdominal segments (Hernandorena, 1979; 1985).

It appears that at least the thoracic and genital (and possibly abdominal) segments can produce both phyllopod and genital structures. This may reflect a more primitive condition, perhaps exemplified in the cephalocaridan Hutchinsoniella which carries two ovisacs that are attached by a short stalk to the limb rudiments on the genital segments.

CHAPTER EIGHT

STRUCTURE OF THE DORSAL THORACIC SETAE AND THE CONNECTION OF THEIR NEURONS TO THE C.N.S.

Introduction

The structures of many cuticular receptors in all the major groups of Arthropods has been well studied (Bullock and Horridge, 1965; Thurm, 1965; McIlver, 1975). The various classifications of receptor types have been derived mostly from studies of insects, and the majority of crustacean cuticular receptors that have been described are those of decapods. There have been very few studies of such receptors in non-decapod Crustacea. Strickler and Bal (1973) described the structure of the setae on the first antennae of the copepod Cyclops and Tyson and Sullivan (1979) made a similar study of setae on the same appendage of Artemia. Other antennal cuticular receptors in Artemia have been investigated by Wolfe (1980) in a study of the frontal knob, a protruberance which develops on the second antenna of the male. Non-antennal cuticular receptors of Artemia have been described in two studies by Tyson and Sullivan (1980 and 1981); on the setae of the adult trunk segments and possible sensilla on the surfaces of the mandibles. None of these studies in Artemia provides detailed information about the ultrastructure or innervation of the sensory processes, as much of the data is derived from scanning electron

microscopy (the last two studies mentioned above use this technique exclusively).

This chapter presents the results of a detailed study of the ultrastructure of the dorsal thoracic setae of Artemia and their innervation.

Results

In a developing thoracic segment the dorsal setae are first externally visible as small cuticular protrusions on either side of the dorsal midline (fig. 8.1). They appear at pre-stage 1 of segment development (undifferentiated mesoderm band stage). As the development of the segment progresses and the ventral phyllopods grow and differentiate, the dorsal setae also grow, from being slight bumps in the cuticle in pre-stage 1 to setae 30μm long in the stage 6 segment (fig. 8.2).

General morphology and position of the dorsal seta

The dorsal seta has a tapering cuticularised shaft which contains two cilium-like dendrites derived from a pair of neurons N1 and N2. The somata of these neurons lie on the inner surface of the dorsal epidermis, slightly ventral and posterior to the seta itself (fig. 8.3).

Around these two dendrites is wrapped a process from a shaft-forming cell, which extends out into the seta. The cell body of this cell lies within the haemocoel on the dorsal side of the two neuronal somata and encloses the two terminal neurites near their point of emergence from these somata (fig. 8.3).

There are two epidermal cells that together form the inner region of the socket of the dorsal seta (socket cells 1 and 2), and there are two or possibly three further cells which encircle these inner cells and complete the socket (fig. 8.3), these are the companion cells. The dorsal seta projects from the dorsolateral epidermis near the region of the segment where there is a constriction (fig. 8.2). The socket of the seta lies in the epidermis above the pericardial haemocoel, just above the dorsal longitudinal muscle (fig. 8.4). The cell body of the shaft-forming cell lies within the pericardial haemocoel on the external side of the longitudinal muscle, but the somata of neurons N1 and N2 lie within the perivisceral haemocoel (fig. 8.4) on the epidermis. Terminal neurites project dorsally from these neurons, around the external side of the longitudinal muscle, through the shaft and socket cells into the seta where they have cilium-like dendrites (fig. 8.3).

The socket cells

The two socket cells are approximately equally-sized and are closely apposed to each other (fig. 8.5). Socket cell 1 completely encloses the shaft cell process (and dendrites) and itself forms a short process on its

apical side which ends at the epidermal surface (fig. 8.6). The second cell (socket cell 2) is flattened against against the first, on the side which encloses the shaft cell process (fig. 8.5). Socket cell 2 encircles the short apical process of socket cell 1 (fig. 8.6), and thus forms most of the external ridge of the socket (fig. 8.3).

The companion cells

There are two or possibly three epidermal companion cells. Their nuclei are at the same level as those of the socket cells. Their apical surfaces extend around socket cell 2 (fig. 8.6) and join together at the epidermal surface to completely surround it.

The shaft cell and axons

The shaft cell lies below the socket cells, within the pericardial haemocoel and encloses the two terminal neurites from neurons N1 and N2 (fig. 8.6). Both neurites contain many mitochondria (figs. 8.5 and 8.6) and their cytoplasm is more electron dense than that of the shaft cell (fig. 8.6). An extension of the shaft cell containing the neurites is enveloped by socket cell 1. This shaft enters the socket cell near its basal surface, at a shallow angle almost parallel with the epidermal surface (fig. 8.3). While the cytoplasm of the shaft cell body is relatively electron lucent compared to the neurites, just as it enters the socket cell its appearance changes. The shaft cell cytoplasm in this region is more electron dense and contains bundles of electron-dense fibrous material some of which is

closely associated with the membranes of the neurites (fig. 8.7), this is known as scolopale material. There is a basal-body in the cytoplasm of the shaft cell, near the neurites, at this level (fig. 8.8). The longitudinal axis of the basal body is parallel with that of the neurites. Near the middle of the socket cell, the shaft cell process containing the neurites turns through a sharp angle towards the epidermal surface (fig. 8.3). At the turning point of the shaft the two neurites become cilium-like dendrites (fig. 8.9) and it is these, enclosed by the shaft cell process, that project outwards through the socket cell and into the seta (figs. 8.3, 8.5 and 8.6). At this level, where the neurites become cilium-like dendrites, the scolopale material in the shaft cell cytoplasm loses its association with the neurite membrane and becomes located in the outer part of the shaft (figs. 8.5 and 8.9). There is a second basal-body in the cytoplasm of the shaft cell, near the epidermal surface (fig. 8.10).

Cell bodies and proximal axons of N1 and N2

The somata of neurons N1 and N2 lie on the epidermis just ventral to the pericardial haemocoel (figs. 8.11 and 8.12). The cells are elongate, bipolar neurons with distal neurites and proximal axons. These axons emerge from the neuronal somata and grow a short distance ventrally until they reach the level just dorsal to the phyllopod bud, where a dorsoventral muscle bundle is inserted. This muscle stretches between the ventral epidermis and dorsolateral epidermis (fig. 8.11). At the dorsal insertion of this muscle, the epidermal cells are elongated in the direction of the muscle's long axis and the axons of N1 and N2 grow along the epidermal cells (fig.

8.12 b and 8.13) and onto the muscle (fig. 8.11). In the specimen illustrated, two epidermal cells were contacted (E1 and E2) before the axons reached the dorsoventral muscle. The axons extend along the muscle towards the ventral surface of the segment (fig. 8.11) and at the point where the muscle passes over the region where the TP axons are found, the muscle comes into contact with a cell (C2) provisionally identified as mesodermal cell. The axons leave the dorsoventral muscle here and turn into a channel formed by the mesoderm cell C2 (fig. 8.14 a and b). They are thereby channelled into the space between the C2 cell and its neighbour C1, where the terminal pioneer axons are located (fig. 8.14 c). The axons of N1 and N2 have thus grown along the posterior edge of the dorsoventral muscle which is found in the posterior of the stage 2 segment.

The axons of dorsal seta neurons N1 and N2 are pioneers

The pair of axons that stretches from the somata of N1 and N2 on the dorsolateral epidermis, to the TP pathway on the ventral surface of the larva, are joined by later-differentiating axons. For example, in the stage 3 segment, there are three axons in this pathway, at various levels near the ventral surface (fig. 8.15), and at later stages these are joined by many other axons. The identity of the cells whose axons fasciculate with the N1 and N2 axon pair has not been investigated.

Discussion

The external surface of Artemia and all other arthropods is protected by a chitinous cuticle. These animals consequently possess special receptors to receive external stimuli which impinge on the exoskeleton. The specialised cuticular receptors which are sensitive to external forces are called cuticular mechanoreceptors or sensory hairs. The dorsal setae of Artemia come into this category. The analysis presented here of the ultrastructure of these setae is the first detailed description undertaken of a branchiopod mechanoreceptor.

Ultrastructure of the dorsal seta neurons

Most studies of cuticular receptors of Artemia have described only the external features, using scanning electron microscopy. Such a description of the dorsal setae is given by Tyson and Sullivan (1980) in a paper which describes the dorsal and ventral setae found on the adult trunk segments. The present study confirms the suggestion made by Tyson and Sullivan; that these setae have a sensory function, and specifically demonstrates that they are cuticular mechanoreceptors. The external morphology of antennular sensilla of Artemia has also been described by the same authors (Tyson and Sullivan, 1979), who found two different types of sensilla, those with pores which are possibly chemosensory and other, longer sensilla which they suggest could be mechanosensory. Neither of the two types externally resembles the dorsal setae and there were no ultrastructural details available for comparison. There is however, one study by Wolfe

(1980), which does include some ultrastructural information about a cuticular mechanoreceptor of Artemia. He described the two types of processes on the frontal knob, (a protrusion located on the antenna of the adult male). The knob bears numerous small, cuticular uninnervated processes and also cuticular mechanoreceptors which appear to possess some structural similarities to the dorsal setae. The latter were shown by Wolfe to contain four sensory dendrites each with a 9+0 arrangement of microtubule doublets in contrast to the pair of dendrites seen in each dorsal seta. These four dendrites are ensheathed by a cell containing four fibrous scolopale bundles, which is in turn enclosed by another cell, in an arrangement very similar to that described for the dorsal seta. It is impossible to accurately assess the exact number and arrangement of cells that form this frontal knob receptor from the data presented by Wolfe, but it is presumably innervated by four neurons that terminate as cilium-like dendrites, although Wolfe describes only a single neuron.

The primary requirement of a mechanoreceptor is that the adequate mechanical stimulus must ultimately cause a depolarising receptor potential in the receptor cell. In the simplest systems this may be achieved by mechanical deformation of the receptor cell leading to an increased permeability of the membrane to ions (Loewenstein, 1965). This may occur for instance in the vertebrate Pacinian corpuscle. There are a group of mechanoreceptors however, that are characterised by the possession of cilia or cilium-like structures which are believed to act as sensory transducers of the stimulus. The dorsal setae of Artemia each contain two modified ciliary structures derived from the two sensory cells that innervate the

receptor. The central pair of microtubules found in motile cilia are absent in these dendrites which consequently contain a 9+0 arrangement of microtubule doublets. This type of modified ciliary structure occurs in receptors sensitive not only to mechanical but also to photic or chemical stimuli (Thurm, 1968). This implies that the modified cilium may not play a direct role in the process of mechanosensitivity but that its structure may be adapted to a more generalised role in the transduction of a variety of types of stimulus. For mechanoreceptors which contain a modified cilium, significance has been attributed to the orientation and organisation of the basal regions of the cilia, especially with respect to basal bodies and ciliary rootlets. The latter are found associated only with the modified cilia of mechanoreceptors such as the chordotonal organs of arthropods (Moulins, 1976) and the basal body has been postulated (Thurm, 1968) to influence membrane current flow patterns due to a shearing force applied to it by the mechanosensitive apparatus, but no experimental evidence yet exists to support this theory. Further studies involving electrophysiological recordings would also be required to establish the function of each of the two neurons which innervates the dorsal seta. The neurons could be sensitive to movement of the seta in different axes: the anterior-posterior axis and the left-right axis. Or alternatively, one neuron may be stimulated by anterior deflection and one by posterior deflection of the seta.

The dorsal setae of Artemia possess a structural feature that is found in all presumed crustacean mechanoreceptors and proprioreceptors, and is always lacking in presumed chemoreceptors. This characteristic feature is

the presence of scolopale material (Ball and Cowan, 1977). This material consists of electron-dense fibres arranged longitudinally around the dendrites of sensory neurons associated with these crustacean receptors (Mill and Lowe, 1973). In the dorsal seta of Artemia the scolopale material is formed by the shaft cell and is grouped into bundles of fibres surrounding the two cilium-like dendrites within the cuticular seta. These fibrous scolopale bundles merge to form a continuous sheath (the scolopidium) around the dendrites below the cuticular surface, in the region where the distal neurites of the two sensory neurons become cilium-like dendrites. The function of the scolopidium and bundles of scolopale material which surround the sensory dendrites is not certain but it seems likely that they provide a rigid support around the fine sensory processes projecting into the seta and maintain the seta as a stiff hair. The arrangement of scolopale material in Artemia (cuticular mechanoreceptor) is the simplest so far described for any crustacean dorsal setae.

The dorsal seta neurons as pioneers

The proximal axons of the dorsal seta neurons pioneer a route towards the ventral surface of the segment and fasciculate with the TP axons. These are the first neurons to be described which serve a sensory function and also establish a nerve pathway. The nerve that is pioneered by these neurons has been described by Benesch (1969) who called it the dorsal nerve. It is largely a motor nerve which innervates the dorsoventral muscles and also the ventral and dorsal longitudinal muscles which will eventually run the length of the thorax. He also noted that the

longitudinal nerves in the abdomen, where there are no ganglia, each have a dorsal nerve branch per segment. These are presumably pioneered by the neurons of the dorsal setae which develop on the abdominal as well as thoracic segments. Axons from the dorsal seta neurons grow out ventrally over epidermal cells and then onto the posterior edge of the dorsoventral muscle which they follow towards the ventral midline of the segment. The point at which they turn off the epidermis onto muscle is where two epidermal cells E1 and E2 are elongated ventromedially presumably due to tension exerted by the muscle fibres inserted onto them. In the one specimen studied, the axons contacted both E1 and E2 before joining the muscle. Whether this pattern is exactly reproduced in every segment requires further investigation, E1 and E2 may be special guidepost cells for the pioneering axons. The same question arises for the presumed mesoderm cell C2 which appears to direct the axons off the muscle towards the TP axons. As was observed for the TP axon branches which pioneer the commissures, the dorsoventral muscles appear to have an important function in the guidance of dorsal seta axons. The posterior edge of the muscle group may be preferentially adhesive for the growth cones of the axons. Whatever the cell surface property is that makes this muscle a suitable surface over which the axons will grow, similar properties may be associated with the cells E1, E2 and C2 which are associated with the ends of the muscle. Peripheral pioneer neurons in insects have not so far been demonstrated to use muscles as guidance for growth towards the C.N.S. Studies of the growth of pioneering axons in the appendages (limb-bud, antenna, wing, cercus) of insects, have shown that they navigate over the basal lamina or epidermis, sometimes

contacting other neurons but not muscle. However, pioneering motor neurons in the grasshopper have been shown to use muscle pioneer cells for guidance as they grow out of the C.N.S towards the appropriate muscle (Ho et al. 1983).

CHAPTER NINE

GENERAL DISCUSSION

The study presented in this thesis of the early development of the thorax and the nervous system of the brine shrimp, contains the first detailed description of the axons of pioneer neurons in the thorax of a non-decapod crustacean and shows how their growth relates to the overall development of the segment.

Segmentation in the thorax

Although the thoracic segments of Artemia develop in an anteroposterior sequence, it is not possible (without further experimental investigation) to determine the segment boundaries that define significant developmental units. The main ways in which segment boundaries have been traditionally defined in the larvae and adults of arthropods have been with the use of structural criteria. In the present study a definition of the segment was chosen which comes into this category; the boundary between segments was defined as the posterior edge of the ventral insertions of a bundle of dorsoventral muscles. In other arthropods, where segments in the adult are fused, the original segmental pattern of the animal is known from embryological studies where the segment primordia are clear. However, implicit in this traditional anatomical approach to segmentation is an assumption that there is a direct developmental correlation between the

embryonic primordia and the adult segments (fused or not). Such a correlation might be assumed for instance in Artemia between the pairs of mesoderm bands with their associated ectoderm and the mature thoracic segments. Such assumptions have been challenged in Drosophila at least, through the use of more modern techniques to follow the lineages of particular cells and the pattern of expression of genes concerned with segmentation (the bithorax complex). It has been discovered that one of the first steps in segmentation of Drosophila is the definition of 14 domains in the embryo, each of which encroaches into adjacent segments later in development. These domains have been termed parasegments and are the fundamental units of pattern formation in the ectoderm and mesoderm of Drosophila (Martinez-Arias and Lawrence, 1985). The borders of the parasegments have been found to be identical with the anterior-posterior compartment borders within segments (Martinez-Arias and Lawrence, 1985). It is not known, in the light of this new evidence, whether the true segments defined in the adult have any importance as units of design during development.

Whether there is a unit equivalent to the parasegment in crustacean development is not known, but a cell lineage study of the ectoderm of a crustacean Diastylis rathkei, has shown that the germ band of this animal is produced from lines of cells, each line giving rise to a well-defined, apparently parasegmental region which will contribute to pieces of appendages from neighbouring segments in the adult (Dohle, 1976). A cell lineage study to follow the fate of the progeny of all the cells of a pair of mesoderm bands and associated ectoderm in Artemia, could determine

whether this primitive crustacean also has a parasegmental unit of development.

Segmentation in the nervous system

The segmental organisation of Artemia and all other arthropods is reflected in the structure of the nervous system. The chain of pairs of ganglia in the thoracic segments of Artemia clearly shows this pattern. In many other adult arthropodan systems however, there is fusion of the embryonic neural primordia, such as in the ventral thoracicoabdominal ganglion of adult Drosophila. In this case, the underlying segmental structure of the neuropile of the fused ganglion can be revealed by monoclonal antibody labelling (Ghysen et al. 1985). The basic segmental unit of the nervous system has been assumed (once again, on purely anatomical grounds) to be a pair of ganglia, joined by anterior and posterior commissures. This assumption has been investigated and reconsidered in a manner comparable to that just described for the mesoderm and epidermis of Drosophila. By the use of fluorescent antibodies raised against protein products of the bithorax complex, it has been demonstrated that the developmental unit of the nervous system overlaps two consecutive segments in a manner comparable to the parasegment described earlier (White and Wilcox, 1984). This is perhaps not surprising as both the epidermis and the neural ectoderm are ectodermally derived. This result has been confirmed by Teugels and Ghysen (1985) who analysed the effects of different bithorax complex mutations on nerve fascicles within the C.N.S. of Drosophila. They demonstrated that the genes of the bithorax complex act on segment-

length units of the nervous system whose boundaries are found to be at the anterior edge of the posterior commissure of a pair of ganglia. The neuronal developmental unit is thus composed of cell bodies and fibres from different ganglia in adjoining segments. It is not possible to propose a direct correlation between the parasegmental domains of epidermal segmentation and these domains within the C.N.S., as the compartment boundaries that delineate the former do not appear to be significant in the nervous system (Teugels and Ghysen, 1985).

It is possible that other arthropods such as the grasshopper and the crayfish (a decapod crustacean) have a comparable parasegmental unit of development in the formation of their thoracic nervous systems, as they have been shown to be virtually identical to Drosophila in terms of their identified cells and processes in the embryonic C.N.S. (Thomas et al. 1985). Artemia however, does not have this same assembly of cells and processes in its embryonic C.N.S. It has been shown in the present study to form the early framework of its thoracic nervous system from a set of pioneer neurons that are different to those described in the grasshopper, crayfish and Drosophila.

Pioneer neurons in Artemia

The TP neurons of Artemia pioneer the first pathways of the longitudinal connectives and transverse commissures in the thoracic segments. This role is unique among the pioneer neurons so far described in arthropods and is also the most extensive, the TP neurons being involved in all eleven

thoracic segments and probably the genital and abdominal segments too. In contrast, the first pathways of the connectives and commissures in the three thoracic segments of the grasshopper are pioneered by a variety of neurons that are arranged on a segmental basis. A group of seven cells called midline precursors which lie along the ventral midline of each thoracic segment are particularly important early pioneer neurons (Bate and Grunewald, 1981). The situation in the grasshopper is made even more complex by the fact that each connective, commissure, neuropile tract or peripheral nerve is composed of many distinct fascicles, each of which is pioneered by a separate axon and forms a labelled pathway that is selectively recognised by other axons (Goodman et al. 1984). The first three longitudinal axon fascicles in the grasshopper embryo initially contain the axons of seven identified neurons (three midline precursor cells and four others). Whether the longitudinal and transverse nerve pathways in Artemia also exhibit a comparable degree of subdivision into fascicles in the mature segment was not elucidated in the present study. It is probable that the axons of the TP neurons pioneer one particular early set of longitudinal and transverse axon fascicles which enables certain neurons to be associated together on an inter-segmental basis. As more neurons develop and initiate axonogenesis, this original framework formed by the TP axons may be elaborated by the addition of further distinct fascicles pioneered by other neurons on a segmental basis. Further studies of later stages in thoracic segment development are needed to investigate these possiblilities in Artemia. As well as being a highly economical method of pioneering connectives and commissures, the long medially branching axons that stretch from the head to the TP cell bodies in the telson, may

perform another particular function for the animal. Where neurons run the whole length of the nerve cord in other invertebrates they are usually involved with whole body responses to stimuli. For example, withdrawal responses of worms or tail-flick responses of lobsters (Bullock and Horridge, 1965). The TP neurons of Artemia may serve a similar role in the coordination of a whole body response, but this remains to be investigated.

The pioneer neurons of the dorsal setae in Artemia are also unique among pioneer neurons so far described in arthropods. Firstly, anatomical evidence suggests that these neurons have a sensory function as well as a role in pioneering a nerve pathway. Although many other peripheral pioneer neurons of insects are presumed to be derived from sensory neurons, most have since lost that function (Keshishian, 1980). Secondly, the dorsal nerve pathway that is laid down by the dorsal seta neurons is the first peripheral nerve to be pioneered in the developing thoracic segment and is connected with the TP axons before the transverse commissure pathways are complete. This situation differs from that described in the grasshopper, where the connective and commissure pathways are linked up before peripheral nerve pathways fasciculate with them.

The small, cuticular, microtubule-filled peg that projects exteriorly from TP2 in Artemia possibly indicates that the TP neurons were/are also sensory neurons as well as pioneer neurons.

Pathfinding by pioneer neurons

The dorsoventral muscles in the posterior of the developing thoracic segment of Artemia play an important role in the guidance of early pioneer neurons. The axons of the dorsal seta neurons grow down the posterior edge of these muscles towards the ventral midline where they eventually leave the muscle to fasciculate with the TP axons. Also, the branches of the TP axons which grow out to form the commissure pathways do so at the two levels in the segment where there is an edge of the dorsoventral muscle which is inserted onto the ventral epidermis. These axon branches are sometimes in contact with this edge of muscle, especially near the midline where they grow over epidermal cells onto which the muscle is inserted. However, more laterally they grow between ganglion cells which lie ventrally below the edge of the muscle.

It appears therefore that the pioneer fibres may have a selective affinity for the edges of the dorsoventral muscles, certain ganglion cells and epidermal cells. It is possible that particular cell-surface antigens are expressed by all the cells over which particular pioneer axons grow. At this early stage in development there may be a single antigen that is expressed by all the cells associated with a particular pioneer that makes them preferentially attractive for given pioneer neurons. A number of different cell-surface antigens have been revealed in the developing nervous system of the grasshopper and Drosophila using monoclonal antibodies, and in Artemia, Raineri and Falugi (1983) have shown that early neurons and the mesoderm cells associated with them all possess acetylcholinesterase

activity which they propose is a 'label' in the early stages of formation of the nervous system. Further experiments, perhaps using monoclonal antibodies, would be required to definitively identify cell-surface properties concerned with the guidance of pioneer axons in Artemia.

Muscles and muscle pioneer cells

Although muscles have not been shown to be important in the guidance of the majority of early pioneer neurons in the grasshopper, some cells called muscle pioneer cells have been described that have a role in the guidance of some early motorneurons in this insect. Muscle pioneer cells were described originally in the grasshopper embryo by Ho et al. (1983). They found that at the same time as neuronal growth cones are beginning to extend along the ventral surface of the basal lamina overlying the neural ectoderm, mesoderm cells also spread along the dorsal side of this same basal lamina. On the left and right sides of each segment the mesoderm spreads medially as two distinct masses of small cells - the anterior transverse muscle anlage (TMA) and the posterior longitudinal muscle anlage (LMA). Near the anterior margin of the TMA they observed a large mesoderm cell (TM1) which begins to stain with the I-5 monoclonal antibody (Chang et al., 1981), and extends processes in the transverse plane. The 15 monoclonal antibody differentially recognises a variety of pioneer neurons and early axonal pathways in the grasshopper embryo (Goodman et al; 1982). This cell inserts its growth cone-like ends into the ectoderm at regions that seem to be equivalent to future muscle insertion sites; at the ventral midline and on the lateral epidermis. These pairs of

cells in the grasshopper are comparable to the pairs of cells observed in the stage 1 Artemia thoracic segment, which also extend processes in the transverse plane to connect ventral and dorsolateral ectoderm. Pioneers of the longitudinal muscles were not investigated in Artemia, but have been observed in the grasshopper. At the posterior margin of the LMA a single cell enlarges, stains with I-5 monoclonal antibody, and sends one process anteriorly and one posteriorly, these processes span the length of a complete segment but are out of register with the segment borders. The posterior process crosses the segment border and stops at the location in the next posterior segment homologous to the position in its own segment where its anteriorly directed process stops. It is thus possible that these muscle pioneers are organised on a parasegmental basis.

In the grasshopper, motorneuron growth cones seem to use the muscle pioneers as one of their guidance cues. The transverse nerve is pioneered by the growth cones of respiratory motorneurones which use the TM1 muscle pioneer cell as their substratum (Ho et al., 1983). In Artemia it is possible that when the dorsal seta axons first begin to pioneer the dorsal nerve, they grow onto the muscle pioneer cells that are first observed in the stage 1 segment of the thorax. By stage 2, when they reach the TP axons, the dorsoventral muscles are becoming well-differentiated. The outgrowth of pioneering axons from the dorsal seta neurons needs to be studied in the pre-stage 2 segments to verify this possibility.

Muscle pioneer cells have also been tentatively described in the leech and these cells have been shown to be involved in the guidance of early axons. In a recent study, the development of the primary peripheral axon of the dorsal pressure (Pd) neuron was investigated (Kuwada, 1985). This is a mechanosensory neuron, its cell body is located inthe C.N.S. of the leech and it pioneers the pathway of the dorsal posterior nerve. The pioneering axon of this Pd neuron follows the entire length of a putative muscle pioneer cell which stretches between the ventral germinal plate near the ganglion and the dorsal germinal plate. Although this axon is growing out of the C.N.S. rather than into it, this system does appear to be similar to the growth and guidance of the dorsal seta axons in Artemia.

The dorsal seta of Artemia and the evolution of subcuticular mechanoreceptors

Two neurons innervate each dorsal seta of Artemia. As just discussed, these neurons pioneer a nerve pathway to the ventral nerve cord. In addition, they are the sensory neurons associated with a cuticular mechanoreceptor. This study of the ultrastructure of the dorsal seta and the dendrites that innervate it shows it to be the simplest of the crustacean cuticular mechanoreceptors so far described. Comparison with other, more complex crustacean mechanoreceptors indicates that the Artemia seta could be the primitive form of the mechanoreceptor of both the cuticular and subcuticular type. The two basic elements of the Artemia receptor; the ciliary dendrite and the scolopale material (fig. 9.1a) are retained but modified and rearranged in the mechanoreceptors of other crustacea.

All crustacean hairs have scolopale structures; Sergestidae (Ball and Cowan, 1977); Neomysis (Guse, 1978); Balanus (Munn et al., 1974); Cyclops (Strickler and Bal, 1973). In all cases except Artemia, the fibrous scolopale material is subcuticular and the sensory processes pass through the ensheathing scolopale bundles and reach directly into the hair. The sensory hairs of these other crustaceans also contain some form of terminal dendrite dilation containing bundles of microtubules. For example in the setae of the first antenna of Cyclops the two sensory dendrites have a 9+0 arrangement of microtubule doublets while they are within the antenna and are surrounded by the scolopale material, whereas in the cuticular shaft of the seta there is a bundle of 100-200 microtubules (fig. 9.1b) (Strickler and Bal, 1973). These crustacean hairs all could be considered modifications of the structure found in the dorsal setae of Artemia with only two notable differences between them. Firstly, in the non-branchiopod crustacean hairs the scolopale material is organised around the sensory processes below the cuticular surface and is not found within the hair shaft. And secondly, there is no terminal specialisation of the cilium-like dendrite in the Artemia setae. It would appear that there has been a modification of the structure of the dendrites within the setae of these more advanced crustaceans, which perhaps confers some greater sensitivity to these mechanoreceptors. However, the microtubule-filled dilations of the dendrites do not require the rigid support of scolopale bundles which are now confined to the subcuticular cilium-like region of the dendrites.

Further modification of the organisation of scolopale material in a mechanoreceptor of a more advanced crustacean than Artemia is seen in the structure of a hair on the antennule of the crayfish Procambarus (Kouyama and Shimozawa, 1981, 1982). This hair has a stiff cuticular shaft which does not contain any sensory cell process but is linked by an extracellular chorda (secreted by the scolopale cell) to a scolopidium consisting of a scolopale cell and three sensory cells. In this receptor the scolopale material is once again subcuticular and as usual, it surrounds the (three) sensory dendrites where their structure is cilium-like. However, more distally there are four bundles of scolopale material enclosing microtubule-filled terminal dilations of the sensory dendrites. This arrangement acts as a tension-sensitive transducer leading to the long chorda within the hair (fig. 9.1c). This sensory hair thus exhibits a different method of mechanoreception. The bending of the sensory hair in this case does not result in the direct bending of a sensory dendrite or terminal dilation of a dendrite. Instead this movement results in tension being applied to the scolopidium. This is situated far from the cuticular hair and contains the sensory dendrites.

The structure of this cuticular receptor strongly resembles that of the subcuticular mechanoreceptors known as chordotonal organs found in both insects and Crustacea. These are stretch receptors, often found at joints and they possess no external cuticular specialisation. The scolopidium in the crab chordotonal organ contains two rather than three sensory cells and in place of the chorda in the cuticular hair, the crab joint receptor has a tube and an elastic strand connecting the exoskeleton and the scolopidium (fig.9.1d) (Howse, 1968; Mill and Lowe, 1973).

Consideration of these four types of crustacean mechanoreceptor suggests that two main modifications of the basic structure of a mechanoreceptor similar to the Artemia dorsal seta could have occurred to secondarily derive subcuticular chordotonal receptors from these simpler cuticular hairs. Firstly, the scolopale material became located in a subcuticular position and it acquired a function in addition to the provision of mechanical support to the fine sensory dendrites. This second function was to act as a tension-sensitive transducer. Secondly, the cuticular hair was lost and the subcuticular sensory elements of the receptor became attached to the inner surface of the cuticle instead of projecting into a hair.

Further evidence that the sensory hair containing cilium-like dendrites is a primitive mechanoreceptor type comes from the fact that similar structures are observed in annelids, molluscs and echinoderms (Cobb, 1968).

Summary and phylogenetic implications

The early development of the thorax and the nervous system of Artemia is described and compared with that of other arthropods.

The thoracic segments of Artemia develop in an antero-posterior sequence, as pairs of mesoderm bands are segregated off from the growth zone in the posterior of the larva. These mesoderm bands, with their associated ectoderm grow and differentiate gradually over many larval instars into mature segments. Each segment will bear a pair of ventral appendages called phyllopods, and a pair of mechanoreceptive setae dorsally. Seven

stages are designated in a description of the external development of the segment, with specific reference to the morphogenesis of the phyllopods.

The early framework of the nerve pathways of the C.N.S. and P.N.S. of Artemia is formed by pairs of axons from pioneer neurons that grow out over uninnervated tissue, and are subsequently followed by later differentiating axons. Axons from the terminal pioneer neurons located near the posterior tip of the larva pioneer the longitudinal connectives and transverse commissures of the C.N.S. The longitudinal pathways are completed first, and subsequently the anterior and posterior commissure axons branch medially from them in an antero-posterior sequence as segment development progresses. The two dorsal nerves in the posterior of each segment are the first periperal nerves to be pioneered to the C.N.S., by axons from the pairs of neurons that innervate each dorsal seta. These grow ventrally and fasciculate with the longitudinal terminal pioneer axons.

Ultrastructural descriptions are given of the terminal and dorsal pioneer neuron cell bodies, axons and associated structures. The dorsal seta neurons have a sensory (presumed mechanosensory) as well as a pioneering function, and there are also indications that the terminal pioneer neurons may be derived from the neurons of sensory receptors. Most other arthropod pioneer neurons so far described do not have such a dual function. Also, the ultrastructure of the dorsal setae is uniquely simple compared to other crustacean mechanoreceptors. In the ventral cord the location of the early pioneer neuron cell bodies and the pathways pioneered by their axons are different and far less complex than in decapod crustacea or insects.

These developmental and antomical features of Artemia are consistent with the Artemia's primitive status among the arthropods. More highly-evolved and complex arthropods such as the grasshopper and crayfish do have many features in common with respect to the early formation of their nervous systems and the ultrastructure of their mechanoreceptive cuticular setae. Many authorities believe that the Arthropoda are polyphyletic (Anderson, 1973) and that similarities between the different arthropodan phyla have arisen by convergence. If that is so, it is remarkable to note such similarities between the embryonic crayfish and insect ventral nerve cord. It is also remarkable that the pioneer neurons of the ventral nerve cord in a primitive crustacean are so different to these.

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