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Embryogenesis of an insect nervous system II: A second class of neuron precursor cells and the origin of the intersegmental connectives

By C. M. BATE[1] AND E. B. GRUNEWALD

From the Institut für Biologie 1, Albert Ludwigs Universität, Freiburg and Max Planck Institut für Virusforschung, Tübingen, Federal Republic of Germany

SUMMARY

The intersegmental connectives in the locust central nervous system are initiated by the axons of early differentiating neuron trios. Using a combination of electron microscopy and fluorescent dye injection we have shown that the axons of these cells grow out anteriorly and posteriorly in each segment along a basement membrane, and link together at the segment borders to form continuous longitudinal pathways on each side of the developing nervous system. These early neurons are the progeny of a second class of precursor cell, the midline precursors, which are distinct from the segmental neuroblasts. Like the neuroblasts, the midline precursors are arranged in a standard segmentally repeated pattern. This and the standard pattern of axon outgrowth in different segments suggest that the nervous system develops to a common, segmentally repeated programme.

INTRODUCTION

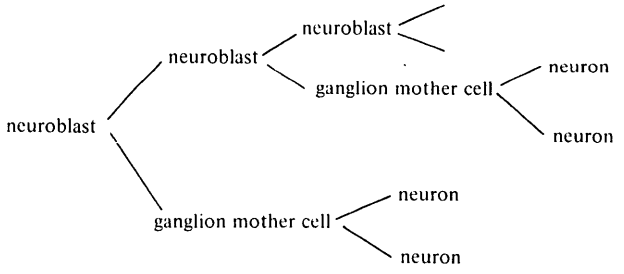
How is axon growth so organized that regular nerve pathways are set up in a developing nervous system? In insects the development of the central nervous system begins with the separation of a layer of neuron precursor cells from the cells of the embryonic epidermis. These precursor cells then generate families of prospective neurons, and a set of such families cooperates to form a segmental unit of the nervous system, a ganglion (Bate, 1976*a*). The first sign of the differentiation of each of these units is the development of a simple pattern of nerves produced by the axons of early differentiating neurons: a rectangle of growing nerves which consists of two transverse commissures and a pair of longitudinal pathways connecting adjacent segments. This simple, metameric pattern forms the basis of the familiar ladder-like arthropod nervous system.

How is this pattern made? We assume that a crucial phase occurs when the first axons grow out in each segment. These axons navigate in response to unknown cues and grow to form a framework of nerves which, as in the peripheral

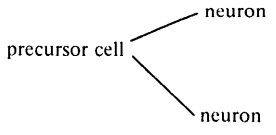
[1] *Author's address*: Department of Zoology, Downing Street, Cambridge CB2 3EJ, U.K.

nervous system (Bate, 1978), is a scaffolding about which subsequent nerve growth is organized. To understand this pattern-forming process better we have looked at the differentiation of early neurons in embryos of *Locusta migratoria* and in this paper we identify the first neurons to produce axons within the central nervous system. The axons of these cells grow anteriorly and posteriorly in each segment to form the longitudinal component of the first nerve pattern, and this intersegmental connection is the pathway about which the future intersegmental connective is organized.

The neurons which produce these first axons have an unusual origin which departs from the lineage of all neurons previously described in the insect central nervous system. In general, central neurons are produced in groups as follows:



The pioneering cells (Weiss, 1941) which we describe here belong to a second group of neurons with a different, shorter lineage:



in which each precursor cell generates only two neurons. We have so far identified seven such precursors per segment, all of which lie on (MP1, 3–6) or near the midline (MP2L, 2R). For this reason, and to distinguish them from neuroblasts proper, we call them midline precursors (MP).

MATERIALS AND METHODS

Eggs and embryos of *Locusta migratoria* were obtained from laboratory stocks in Canberra, Freiburg and Tübingen. Precisely timed embryos were taken from egg pods collected at the time of laying and stored in a water bath at 30 °C. At this temperature embryogenesis lasts 13 days.

Embryos were prepared for electron microscopy as described previously (Bate, 1976*a*). Serial ultrathin sections were mounted on coated slot grids.

Neurons were located in the living embryo under a compound microscope using differential interference contrast illumination, penetrated with a microelectrode and filled with the fluorescent dye, Lucifer Yellow (Stewart, 1978), using methods developed and described by Goodman & Spitzer (1979).

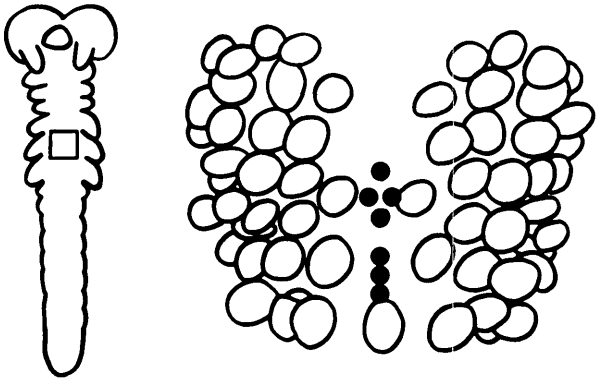


Fig. 1. Map of neuronal precursor cells as seen from the ventral surface of a meso-thoracic segment (indicated by square outline in embryo). Open circles: neuroblasts; filled circles: midline precursors (MPs).

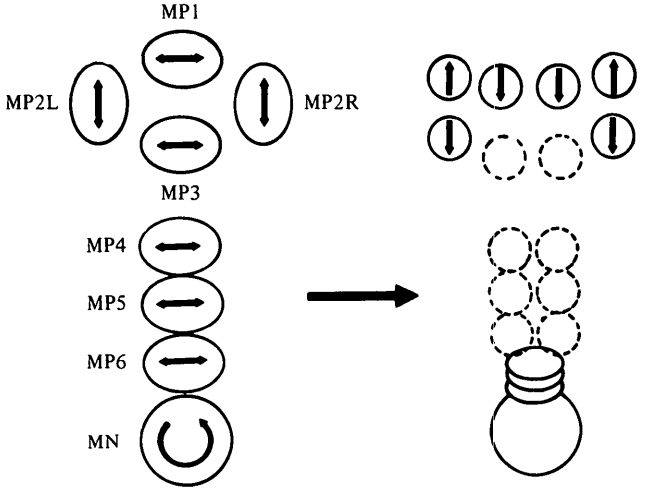


Fig. 2. Diagram to show the arrangement of the midline precursors and their division pattern. Left: seven midline precursors and the single posterior median neuroblast (MN). Arrows indicate single divisions of MPs, repetitive divisions of MN. Right: Products of MP divisions and neuroblast divisions. Progeny of MP 3–6, dotted circles. Progeny of MP1 and 2 and MN, closed circles. Arrows indicate direction of axon growth from MP1 and 2 progeny.

RESULTS

(1) *Precursor cell pattern*

All symmetrically dividing neuron precursors so far found lie on or near the midline (Fig. 1). There are seven MPs in each of the thoracic and anterior abdominal segments. In this paper we deal exclusively with MP1, 2L and 2R and their progeny (Fig. 2). Subsequent papers deal with MP3 and the development of the two neurons which it produces (Bate, Goodman & Spitzer, 1981; Goodman, Bate & Spitzer, 1981).

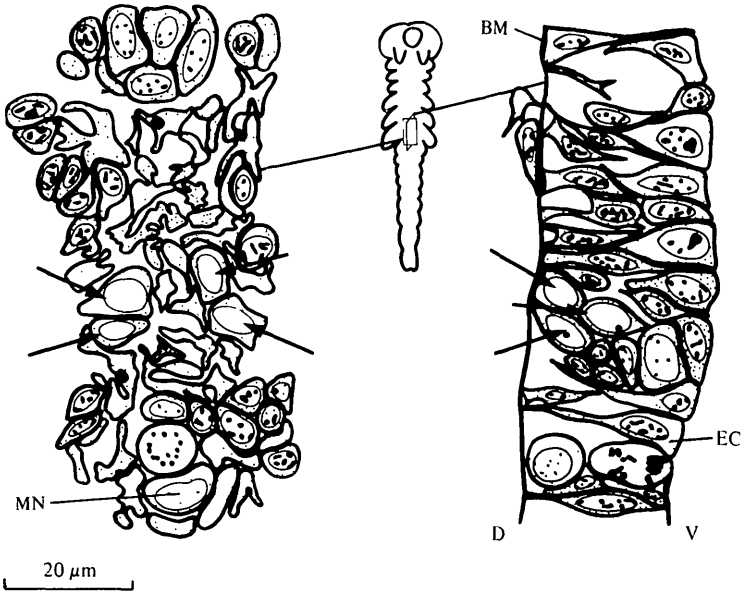


Fig. 3. Camera-lucida drawings of alternative planes of section through the midline of the metathorax at 90 h to show cell trios and associated cells and structures in the neuroectoderm layer. Left: frontal section. MN: median neuroblast. Two cells each from right and left trios present in this plane arrowed. Right: sagittal section. D: dorsal, V: ventral, BM: basement membrane, EC: epidermal cell. Cell trio arrowed.

(2) *Origin of precursors and sequence of cell divisions*

The two MP2 cells in each segment, which can be recognized in sections as enlarged cells lying dorsal to the surrounding neuroblasts, appear simultaneously on either side of the midline at about 60 h in the thorax. MP1 appears about 6 h later, slightly anterior to the two MP2 and exactly on the midline. Each of these three precursor cells seems to arise by simple enlargement among the cells of the surrounding epidermis. The sequence of division follows the sequence of origin. The two MP2 divide with the spindle oriented obliquely to the embryonic surface. MP1 first appears after this division, and after enlarging divides with the spindle axis parallel to the surface to produce two daughter cells, one on either side of the midline. In this way a cluster of three prospective neurons is generated on each side of the midline between neuroblast rows 4 and 5 (Bate, 1976*a*) (Fig. 3).

(3) *Axon pathways from the cell trios*

Ninety-six hours after egg laying, neurons differentiating in the ganglia of the thorax and anterior abdomen (development of the abdomen occurs in an antero-posterior sequence) have begun to produce axons. The growing nerves are pro-

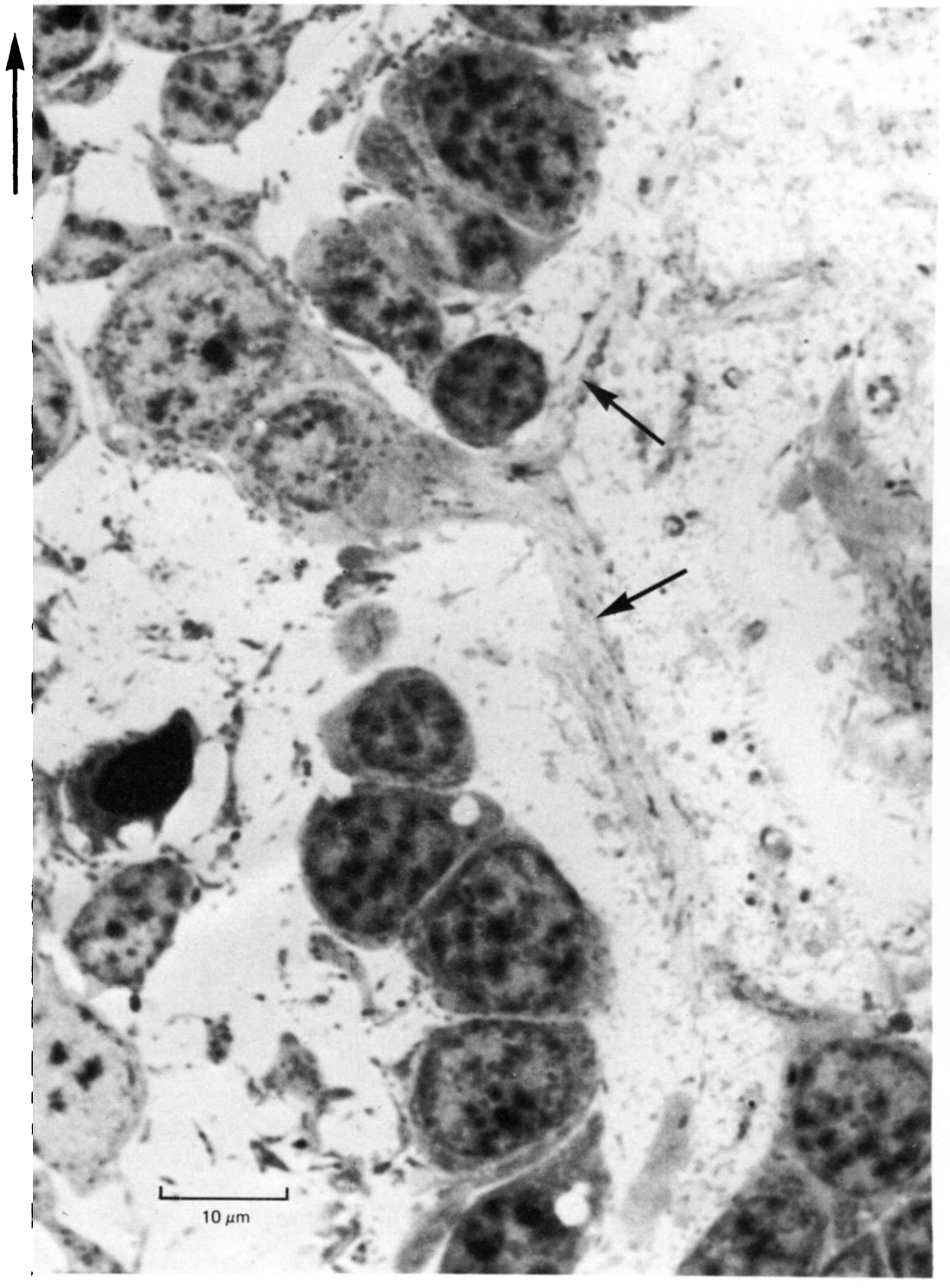


Fig. 4. Semithin frontal section through the right half of a metathoracic ganglion at 96 h. Pair of cells from right cell trio are visible, together with longitudinal axon pathways (arrowed). Long arrow points anteriorly.

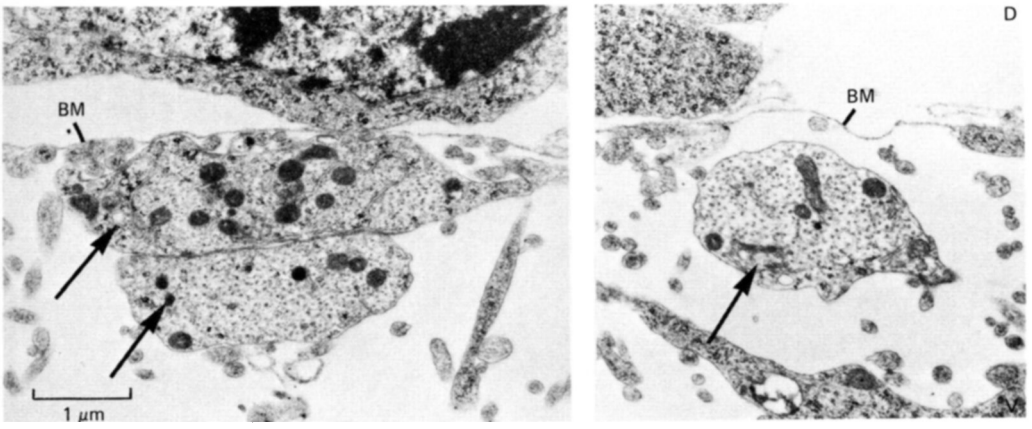


Fig. 5. Electron micrographs of transverse sections through the metathoracic ganglion, posterior (left) and anterior (right) to the midpoint of segment at 96 h. Two axons run posteriorly from the midpoint of the segment (arrowed left) and a single one anteriorly (arrowed right). BM, basement membrane; D, dorsal; V, ventral.

duced both by the progeny of neuroblasts and by the trios of cells in the middle of each segment. Serial light microscope sections made at this stage show that the axons from the central cell trios have grown initially dorsally and then separated into two longitudinal pathways which run posteriorly and anteriorly to the segment borders beneath a dorsal basement membrane (Fig. 4). It is important to note that axon growth in the embryonic insect nervous system takes place within a neuroectodermal cell layer consisting of epidermal cells, neuroblasts and neurons, which is bounded dorsally by a basement membrane which separates the embryonic ectodermal and mesodermal layers (Figs. 3 and 5). The axons appear to grow within the clefts which separate the families of cells produced by adjoining neuroblasts. These clefts are initially occupied by the processes of epidermal cells which extend dorsally between the neuroblasts to contact the overlying basement membrane.

Serial ultra-thin sections cut frontally through the ganglion confirm that each cell trio forms two pathways which run longitudinally in opposite directions from the middle of the segment. Transverse sections show that the anterior pathway is followed by a single axon, the posterior by two axons which run together along the dorsal basement membrane (Fig. 5). Combining our results from the light microscope, which show the origin of each of the three cells, with those from the electron microscope, which demonstrate the origin of each of the three axons, we find the following: each MP2 produces a more dorsal and a more ventral neuron. The axon of the more dorsal cell runs posteriorly, that of the more ventral, anteriorly. The single daughter of MP1 on each side of the midline produces an axon which combines with that of the more dorsal MP2 daughter and runs posteriorly (summarized in Figs 2 and 6).

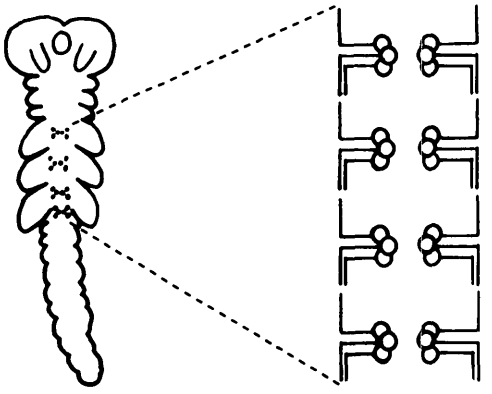


Fig. 6. Diagram to illustrate the pattern of axon pathways formed by the cell trios present on the midline in each segment.

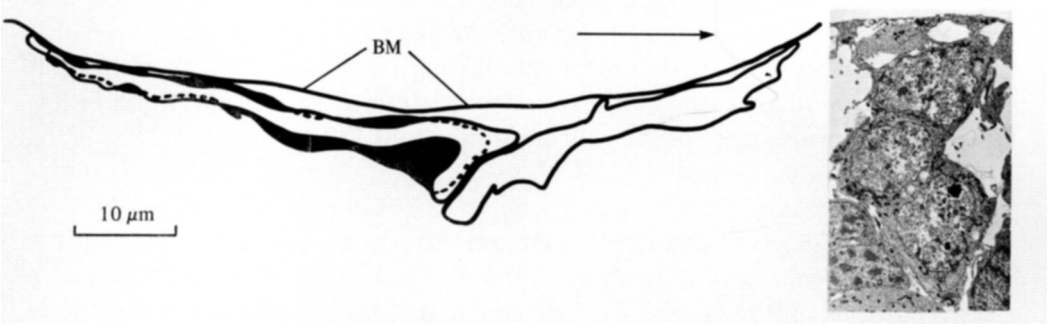


Fig. 7. Reconstruction of axon pathways from a cell trio in the mesothorax at 90 h. The plane of section is sagittal and the inset shows an electron micrograph of the cell trio concerned. The most ventral cell sends an axon anteriorly along the basement membrane (BM), the two more dorsal axons grow posteriorly. Arrow: anterior.

(4) *Axon initiation*

The cell trios begin to put out axons at about 80 h in the thoracic segments and then in an anteroposterior sequence in each of the abdominal segments in turn. At the earliest stage in this process which we have so far observed, the outgrowing axons of the two MP2 daughter neurons emerge together and form an expanded region dorsolaterally to the neurons themselves, against the basement membrane. We find that the most dorsal and youngest of the three cells, the single daughter of MP1, lags behind the other two in producing an axon. At a slightly later stage a single axon extends anteriorly along the basement membrane, and two posteriorly (Fig. 7). There are no other axons present in the system at this stage.

(5) *Segment border*

About 16 h later, at 96 h (in the thorax), both anteriorly and posteriorly growing axons have reached the segment border. This they cross, the axons

from neighbouring segments growing past each other, with the single axon of the more posterior segment growing medially to the two from the more anterior in the cases which we have so far observed. Although the axons of many other differentiating neurons are present within each segment by this stage, there are none in the region of the segment border. The first link between adjacent segments of the nervous system is established by the three axons which we describe here.

(6) *Further development*

To study the further development of the axons beyond the boundaries of their own segment, we filled the neurons with a fluorescent dye, Lucifer Yellow (Stewart, 1978), at different stages of development. The neuron trios lie immediately beneath the limiting membrane on the dorsal surface of the nervous system. If an embryo is freed from yolk and pinned out with its dorsal side uppermost, the neurons are easy to identify with a compound microscope (Fig. 8) and accessible for the injection of dye through a microelectrode. Using this method we were able to confirm the early pattern of axon growth and follow the further development of the neurons until about 132 h.

(7) *Cell coupling*

The two neurons whose axons grow posteriorly lie most dorsally, and it is simplest to inject one of these more exposed cells directly with dye. However, in nearly all cases all three cells fill with dye when one is injected and because of this coupling the pathways of all three axons are usually revealed in a single experiment. In early development the first neurons are also coupled via their axons with other cells in the same segment, in particular with a conspicuous neuron pair whose cell bodies consistently fill in the posterior half of the same segment (Fig. 9). Later in development, when the growing axons have reached adjacent segments, faint fluorescence indicates dye coupling between equivalent cell trios in neighbouring ganglia. By 132 h this coupling may be detected in up to four ganglia in either direction from the segment where the injection has taken place (Fig. 9).

(8) *Growth beyond the segment border*

As expected, dye-injected axons are seen to reach the segment border at about 96 h (thorax), cross it and enter adjacent segments. Dye coupling between equivalent cells in adjacent segments complicates the question of how far individual axons actually grow. Occasionally however, cells are filled which are uncoupled from adjacent segments and in these cases the axons have extended up to one and a half segments from their point of origin at 110 h and two segments at 120 h (Fig. 10). The axons follow a consistent, curved pathway to the segment border (Fig. 9) and this route is repeated in each segment which they enter, suggesting that they grow along the path offered by their counterparts and

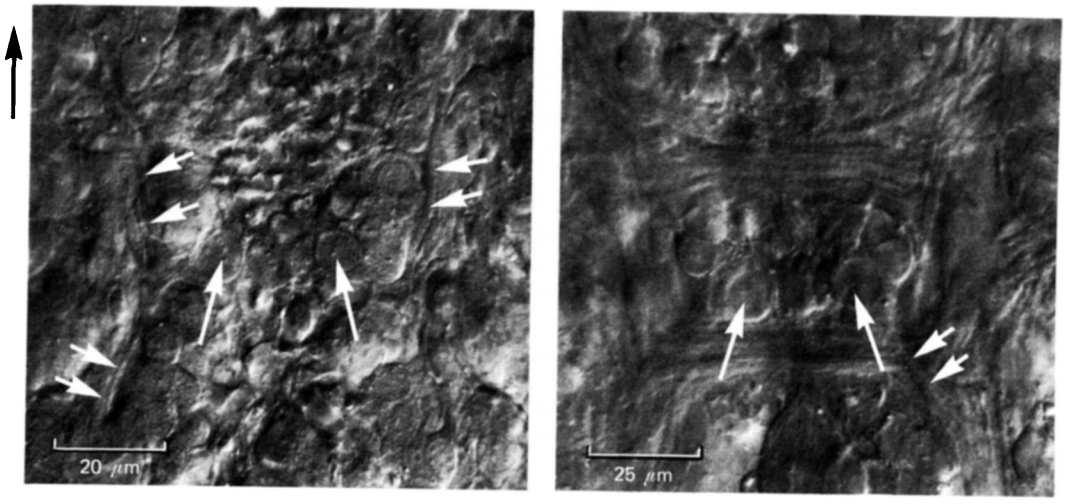


Fig. 8. Micrographs of the dorsal surface of living embryos viewed with differential interference contrast illumination. Metathorax, left 96 h, right 115 h. Large arrows: cells from the central trios, small arrows: their axons. The rectangular pattern of nerves is conspicuous at 115 h. Arrow at left points anteriorly.

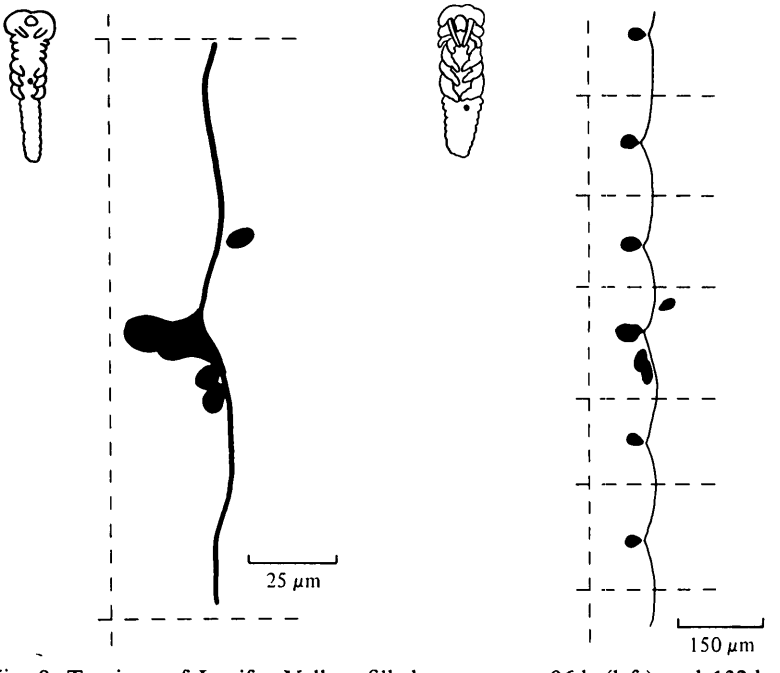


Fig. 9. Tracings of Lucifer Yellow filled neurons at 96 h (left) and 132 h (right). Cells are dye coupled both intra- (left) and intersegmentally (right). Dotted lines: midline and segment borders. Insets: embryos showing injection sites (dots).

Fig. 10. Micrograph of a neuron filled with Lucifer Yellow in abdominal segment 1 at 120 h. Simultaneous white and ultraviolet illumination to show axon extending over two segments from its point of origin. Arrow: anterior, dotted lines: segment borders.

provide a path for other axons entering their own segment. A comparison of the time taken by the outgrowing axons to cross one half of one segment (80–96 h) with the time subsequently taken to grow one segment further (96–110 h) shows that there is a slow and a fast phase of axon extension. We assume that the initial, slow phase is exploratory, and that growth accelerates at the meeting point between axons from equivalent trios in neighbouring segments. The longitudinal pathway is complete and the growing axons can extend rapidly along the track provided by their counterparts in adjacent parts of the nervous system.

DISCUSSION

The fundamental arrangement of nerve pathways in the insect nervous system is a simple one: each segmental ganglion is connected with its neighbours by longitudinal pathways, opposite sides within each segment by paired commissures, and it is within this framework that the differentiation of individual neurons occurs. We have two questions to ask about this nerve pattern: How is it formed and how does it influence the organization of differentiation within the ganglia?

(1) *How is the pattern formed*?

Because of the relatively large size and constant position of the cells concerned we can show that the longitudinal element in the nerve pattern is formed by a linking together of repeated units consisting of equivalent neurons which are generated early in development in each of the future ganglia. The nervous system is therefore assembled segmentally, even where the construction of a finally suprasegmental element, the longitudinal connective, is concerned. Indeed, so far as the early events in embryogenesis are concerned, the nervous system seems to develop to a single, common programme which is repeated in every segment outside the brain.

In each segment we detect a pattern-forming process which ensures that cells in the ventral region of the embryo are assigned to form neuron precursors in a standard arrangement. The pattern of MPs is identical in each segment, that of the neuroblasts nearly so (Bate, 1976*a*). This pattern of precursor-cell differentiation suggests that there is a standard segmental organization of cell determination in the developing nervous system, as in other cell layers such as the epidermis (reviewed by Lawrence, 1973).

In addition we observe a second, not necessarily independent pattern-forming process which is revealed in each segment as the first axons are initiated in the future ganglia. Like the first axons in the peripheral nervous system (Bate, 1976*b*) the first central nerves grow out in an environment which is devoid of other nerves, and the ordered way in which they grow reveals a transfer of pattern from one cell sheet (the neuro-ectoderm) to an axonal layer (the early axons described here) growing through it. In other words, assuming growth is not intrinsically programmed, the cues to which the first outgrowing axons respond represent an existing organization in the sheet of cells through which they grow, and their response to these cues builds a simple pattern of nerve pathways into the embryonic nervous system. Possibly these cues remain available to *all* axons in the developing nervous system; alternatively, there may be a unique transfer of information between the first pioneering axons and the cell sheet through which they grow, so that the first pathways then remain as essential guides for the subsequent organization of nerve growth. Either way, the common repeated pattern of nerve differentiation reveals a standard, segmental organization within the cell sheet through which the first axons grow.

In each segment the first pathways are formed by two sister cells whose axons grow out in opposite directions. It is striking that these two axons are sent out together, encounter the dorsal basement membrane together, and then grow out in opposite directions along it. We can suggest several possible explanations for this pattern of growth. First we cannot exclude that the final structure of each cell may depend on a stringent, intrinsic ordering of axon growth. Alternatively both cells could be completely naive and respond to mirror-image cues which reverse at the middle of each segment, sending the axons in opposite directions. This is improbable, as the environment of both sisters is practically the same at the point at which the anterior/posterior decision is made, that is to say the growth cones of both axons encounter the basement membrane at the same level in the anteroposterior axis. Were the cells equivalent, we would expect chance to determine which neuron sent its axon anteriorly and which posteriorly. Our observations show that it is always the ventralmost cell which sends its axon anteriorly and this indicates that the two sisters are significantly different from one another. We offer a third explanation: that the cells are partially programmed by their developmental history, such that they are mirror images of each other (as in the case of some sister cells in fibroblast and neuroblastoma cell cultures, Albrecht-Bühler, 1977; Solomon, 1979) and in this way they respond in opposite senses to a single, segmentally repeated cue giving longitudinal polarity. Some sort of interaction is required for a transfer of pattern between the neuroectodermal cell sheet and an axonal layer growing through it. We assume that the cue to which the first axons respond is associated with the basement membrane over which they grow, which represents an interface between them and the neuroectodermal cell layer.

(2) *The first nerve pattern and subsequent neuron differentiation*

Do the first axon pathways act as guides for future nerve growth? One of the striking features of axon growth in the developing neuropiles of the embryonic insect nervous system is that it occurs within a space defined by the limiting dorsal membrane and the surface of the neuroblasts, neurons and epidermal cells beneath it, and that this space is devoid of structures other than growing axons and fragments of epidermal cell processes (Bate, in preparation). We have to assume that pathways formed by early axons (including the three described here) do influence the organization of later axonal growth, at least passively, simply because they are one of the few available surfaces over which further axon growth can occur. If this influence is a passive, mechanical one, then we must assume that the surfaces of different axons are similar and that nerve growth over these surfaces is directed by other, unknown cues. If, on the other hand, the surfaces of different axons are distinct, then the distribution of these surfaces will provide a differentiated structure, increasing in complexity as the ganglion develops, within which neuronal differentiation can occur. In this case, early pathways will be significant determinants of ganglionic organization.

Observations made in the course of this work support this view to a limited extent. The fact that we can divide the growth of the first axons into a slow phase, and a second, faster phase in association with other nerves, encourages us to believe that the first axons do indeed act, in this case reciprocally, as guides for the growth of other nerves. In addition the exclusive pattern of dye coupling between cell trios in neighbouring segments, and consistently with particular cells in their own segments implies a selective association between at least some of the cells in the developing nervous system. Preliminary observation of other cells whose axons cross the midline during development shows that these cells, despite the close proximity of many other axons, are at least initially, exclusively coupled with their equivalents on the opposite side (Bate and Whitington, unpublished). There appears to be a selective association between such cells as their axons cross in opposite directions at the midline.

We do not yet understand the particular significance of the longitudinal pathway which we describe. We would expect the segment borders to be important elements in the developing nervous system, as dividing lines between equivalent units of differentiating neurons. In the peripheral nervous system the segment border actually maintains the distinction between these units by acting as a barrier to the growth of sensory axons between them (Lawrence, 1975). In the central nervous system an intersegmental link is formed in sequence as a bundle reflecting the progressive addition of equivalent axons from neighbouring segments. We anticipate that this is the foundation for an orderly distribution of nerves in the future connectives. It is also not clear why the neurons involved in forming this first pathway should have an unusual origin. One simple consequence of their shortened lineage is that the daughters of MP2 produce axons before the progeny of neuroblasts that were generated at the same time as the MPs. It is worth noting that the pattern of divisions which produces the central cells described here is the same as that of the pioneer sensory cells in the appendages (Bate, 1976*b* and unpublished observations). It may be that cells with this origin have a special part in the assembly of a first axon framework in the embryonic nervous system.

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