

Development of a sensory afferent projection in the grasshopper embryo

I. Growth of peripheral pioneer axons within the central nervous system

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SUMMARY

The grasshopper's cercal nerve is established early in embryogenesis by an identified pair of peripheral neurons called the cercal pioneers. Like the peripheral pioneer neurons in other insect appendages, these two cells send their axons from the periphery to the rudimentary CNS and thus lay the foundation for a nerve that will later be followed by a large number of sensory axons. In this paper, cobalt fills of the primordial cercal nerve were used to characterize the disposition of these peripheral pioneer axons within the embryonic CNS. The pioneer axons stained by this technique terminate in ellipsoidal growth cones which have filopodia radiating from the leading edge and a single long terminal filament pointing along the path the axon is taking. The growing axons also bear filopodia along their sides, but these structures disappear as the cells mature. The pioneer axons of the cercal nerve make an abrupt turn where they first enter the ganglion rudiment and join the axons of the primary longitudinal tract. The pioneers then grow along this tract for several hundred microns without forming secondary growth cones or branches. This prolonged absence of central arborization distinguishes the peripheral pioneer axons from the axons of later-arising epidermal sensory neurons.

INTRODUCTION

Axons routinely grow across long distances to reach specific targets in the developing nervous system (Edwards, 1977; Bate, 1978). A great many fibers follow pre-existing nerves, but the earliest axons must pioneer their own routes and navigate a cellular landscape that is devoid of any established neural path. Such cells offer a unique opportunity to study the growth of the axon and the parameters which guide it during ontogeny. In addition, the nerves established by these pioneering axons seem to be an important source of guidance for subsequent fibres. Hence, the paths chosen by pioneer axons may determine the fundamental pattern of nervous system interconnexion.

Insect sensory neurons arise in the epidermis and their axons grow to the central nervous system (CNS) along pre-existing nerves (Wigglesworth, 1953).

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These nerve routes are first established during the embryogenesis of hemimetabolous insects by a unique class of pioneer neurons originally described by Bate (1976*a*). He used electron microscopy to identify the first axon profiles in the antenna and leg of the locust embryo, then located the cell bodies of these pioneer neurons in light microscope sections. The principal nerve trunks in both appendages are founded by discrete, uniquely identifiable pairs of early-differentiating peripheral neurons whose somata lie on the inner surface of the epidermis. Keshishian (1980*a*) has since described an identical pair of pioneer neurons in the leg of the grasshopper embryo, and shown that these two cells are siblings which migrate from the epithelium to the interior of the limb before their axonogenesis begins. Edwards & Chen (1979) have described the embryogenesis of the cricket's cercal nerve, and report that the first cercal axons also derive from peripheral neurons with sub-epidermal somata. The uniformity of these findings, as well as the results presented here, suggests that the peripheral pioneer neurons of the insect embryo are a distinctive class of cells, characterized by both anatomical and developmental features, which play a common role in the construction of a variety of different afferent nerves. A specialized class of early-differentiating peripheral neurons have also been implicated in the establishment of adult nerve routes during the pupation of a holometabolous insect species (Sanes & Hildebrand, 1975).

The peripheral pioneer neurons of the insect embryo have a fundamental position in the organization of sensory pathways. They link the appendages to the appropriate ganglia early in embryogenesis, when the intervening distance is small, then maintain this connexion as the nerve is drawn out to much greater lengths. The nerve established by the pioneer axons then serves as a substrate for the aggregation and centripetal growth of a large number of sensory axons which arise from the surrounding epidermis at later stages in life (Edwards & Chen, 1979; Keshishian, 1980*b*; Toroian-Raymond & Bentley, 1980). The sensory axons of the embryonic cricket cercus fail to aggregate in the usual manner if the portion of that appendage which contains the pioneer neuron precursors is ablated prior to nerve formation (Edwards, Chen & Berns, 1981), which suggests that the pioneer axons constitute an obligatory substrate for the sensory axons to follow their normal paths. Thus, the peripheral pioneer neurons may have a unique and irreplaceable function in the construction of the afferent nerve.

Since previous studies of insect pioneer neurons have been restricted to the periphery and fail to address the axons' central fate, the present paper examines the growth of peripheral pioneer axons once they have entered the CNS. This was accomplished by cobalt filling the cercal nerve of the grasshopper embryo at stages which were sufficiently early in development so that only the pioneer axons had yet reached the ganglion. Cobalt fills performed at a series of different stages chronicle the passage of the pioneer axon's growth cone along its central path. A companion paper (Shankland, 1981) describes the central arrival and

branching of the sensory axons that follow these pioneers. The results presented in both of these papers have been summarized elsewhere (Shankland, 1979).

METHODS

The present study uses embryos of the California grasshopper *Schistocerca nitens*. The eggs were raised on moist sand at 30 ± 1 °C, so that complete embryogenesis lasts 28–30 days. The developmental age of the experimental embryos will be defined as the percentage of this total embryonic period which has been completed, in accordance with the 5% interval staging descriptions presented in Bentley, Keshishian, Shankland & Toroian-Raymond (1979). That paper also gives details for the maintenance and handling of grasshopper embryos, as well as embryology which is pertinent to this text.

To observe peripheral neurons and axon tracts, the unstained, living embryo was dissected from its egg into an osmotically adjusted saline (Bentley *et al.* 1979), then squashed beneath a cover slip and viewed with differential interference contrast (Nomarski) optics on a Zeiss Universal compound microscope. Over 50 embryos between the ages of 30–60% were examined in this fashion.

The central terminals of the cercal afferents were stained by axonal diffusion cobalt fills of the embryonic cercal nerve. The cercus was sealed into a vaseline chamber containing 1% CoCl_2 and 0.13 mg/ml bovine serum albumin (Strausfeld & Obermayer, 1976) after tearing open the bud to expose the nerve within. A human hair tied around the base of the cercus can be used to anchor the vaseline seal and minimize leakage of cobalt into the body cavity. The embryo was then kept for 3–4 h at room temperature (21–24 °C) in a continuously circulated tissue culture medium (Chen & Levi-Montalcini, 1969) consisting of a 5:4 mixture of Schneider's *Drosophila* Medium and Basal Medium Eagle (GIBCO; Grand Island, N.Y.). This procedure occasionally fills the afferents of the neighbouring epiproct nerve (Seabrook, 1968) in addition to the cercal fibres. Cobalt impregnated axons were stained by ammonium sulphide precipitation (Pitman, Tweedle & Cohen, 1972), and the specimen fixed in Carnoy's solution and cleared in methyl benzoate. The cobalt sulphide precipitate was then silver-intensified with the whole mount Timm's method of Bacon & Altman (1977). Ganglia were sectioned (6–40 μm thick) in paraffin, counterstained with toluidine blue, and mounted in neutral Canada Balsam. The descriptions of peripheral pioneer axons presented here are based on fills of 20 different afferent projections.

The Rowell (1963) silver stain was used to ascertain the general structure of the developing neuropil. The abdominal ganglia were examined in serial 10 μm sections at each 5% stage between 35 and 95% of embryonic life, as well as the adult. With the incubation step performed at 60 °C and pH 8.4, both neuropil and nerve tracts were darkly stained in embryos as young as 45%.

RESULTS

Peripheral pioneer neurons

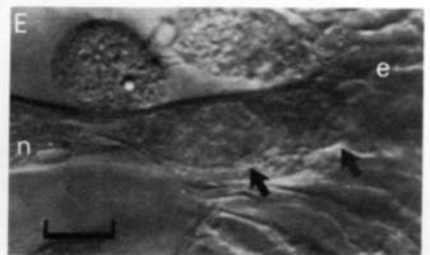
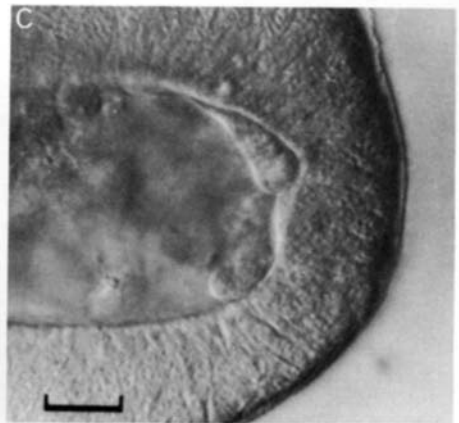
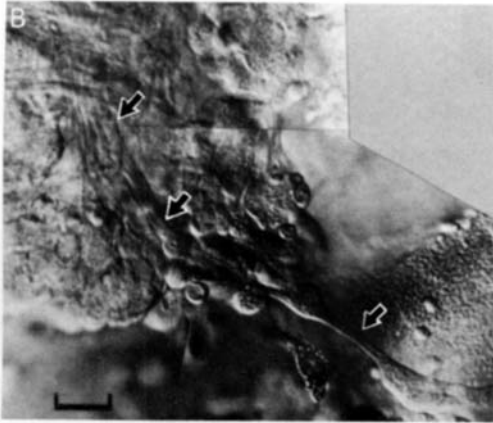
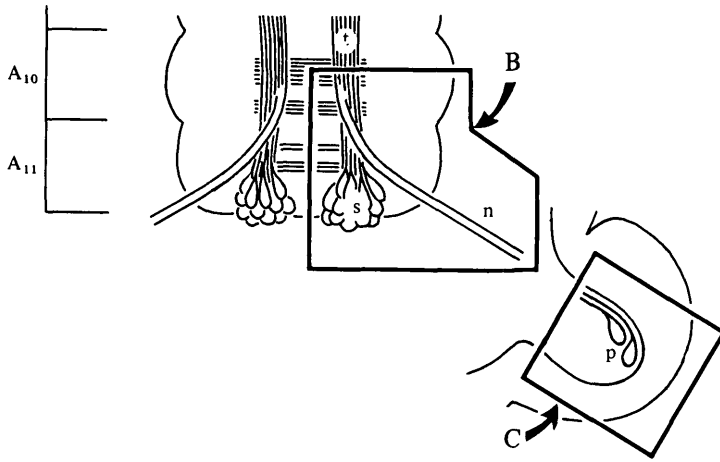
The cerci are bilaterally paired sensory appendages which arise from the grasshopper's terminal (eleventh) abdominal segment. They are first seen at 35% of embryonic life as evaginations of the lateral ectoderm. This ectodermal layer pulls free from the underlying mesoderm and envelopes a hollow space, the cercal lumen, which is continuous with the main body cavity. The ectoderm then differentiates into the epidermis of the mature cercal bud.

Morphologically differentiated neurons are first seen within the cercus at 40% of embryonic life (Fig. 1). Nomarski observations reveal a pair of large (15 μm dia.) neurons lying in a shallow depression on the inner surface of the epidermal wall at the lumen's distal tip. These two cells are readily distinguished from neighbouring epithelial cells by their sharply defined nuclear envelopes and finely textured chromatin (Fig. 1D). Each neuron has a single thick (1–3 μm dia.) axon whose fibrous appearance suggests an organized cytoskeleton, and a tapered axon hillock which lends the cell a characteristic teardrop shape. These two axons grow in unison along the epidermal wall from the cercus to the CNS. Since they are not associated with any other nerve trunks at this stage, these axons are believed to be the pioneering fibres of the embryonic cercal nerve.

The anatomical relationship between the cercal pioneer neurons and the CNS is shown diagrammatically in Fig. 1A. The pioneer axons reach the edge of the CNS, which lies 50 μm from their cell bodies, before the end of the 40% stage and enter the ganglion immediately lateral to a cluster of central neurons whose anteriorly directed axons comprise the primary longitudinal fibre tract (Fig. 1B).

Fig. 1. Cercal pioneer neurons. (A) Diagram showing the anatomical relationship of the CNS (part B) and the cercus (part C) at 45% of embryogenesis. Brackets at the left show the embryonic segmentation of the rudimentary ganglia. p, Pioneer neuron cell bodies; n, cercal nerve; s, somata of longitudinal tract neurons; t, longitudinal tract. (B–E) Photomicrographs of unstained, living tissue viewed with Nomarski optics. B. By 45% the primordial nerve containing the two pioneer axons has become dotted with glial cell nuclei and detaches from the epidermis to span the body cavity. The pioneer axons (arrows) can be traced as they enter the posterolateral corner of the ganglion and turn toward the anterior to join the axons of the longitudinal tract. Scale 20 μm . (C) A pioneer neuron lying in a shallow depression of the epidermis at the distal tip of the cercal lumen. The other member of the pioneer pair is located behind this cell and cannot be seen. The axons arising from these neurons follow the inner surface of the epidermis toward the CNS. Scale 20 μm . (D) The cell bodies of the pioneer neurons (arrows) are readily distinguished with Nomarski optics by their sharply defined nuclear envelopes and finely textured chromatin. Their axons (a) have a fibrous appearance which most likely results from an organized cytoskeleton (40% embryo). Scale 10 μm . (E) As the cercal bud grows, the pioneer neurons (arrows) come to lie against the epidermis (e) in the apical tip of the lumen at the distal head of the cercal nerve (n) (50/55% embryo). Scale 10 μm .

A



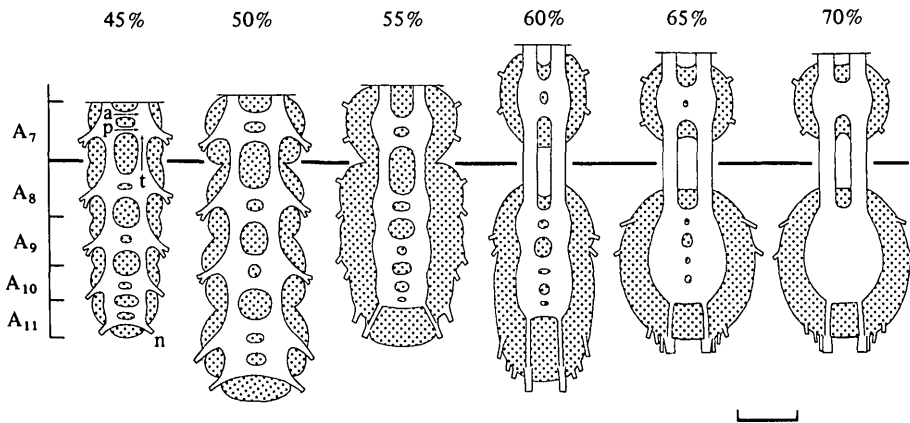


Fig. 2. Fusion of the terminal abdominal ganglion from embryonic rudiments A_8 – A_{11} . The cell body layer is shown by stippling, and the embryonic segmentation (at 45%) is given by brackets at the left. The afferent tract of the cercal nerve (n) is shown crossing the cell body layer. Outlines were reconstructed from silver-stained serial sections. a , Anterior commissure; p , posterior commissure; t , longitudinal tract. Scale $100\ \mu\text{m}$.

The pioneer axons make a relatively abrupt 50 – 60° turn to join these fibres, and proceed to grow anteriorly along the medial edge of the tract.

The nerve trunk established by the cercal pioneers is the embryonic antecedent of the cercal sensory nerve found in the adult grasshopper. (See Seabrook, 1968, for the distribution and nomenclature of the adult nerves.) Putative glial cells begin to enwrap the bare pioneer axons by 45%, and the resultant nerve trunk loses contact with the underlying body wall over most of its length (Fig. 1B). The glial sheath comes to obscure the axons within the living nerve, but the cell bodies of the pioneer neurons can still be seen at the nerve's distal head (Fig. 1E). These somata remain in contact with the most distal portion of the cercal epidermis as this appendage elongates from its rudimentary spherical form into a cone from 45–60%. As a consequence, the pioneer neurons become nestled in the apical tip of the mature cercal lumen. The entire embryo expands to fill the egg during the same period of time, and the cercal nerve is drawn out to 15 – $20\times$ its original length. When the epidermal sensory neurons differentiate at 55–60%, their axons grow from the epithelium into this nerve and follow it across this expanded distance to the CNS.

Although no other pairs of cercal pioneer neurons have been identified by Nomarski observations, other such cells might not have been seen with Nomarski optics if they differentiate at a later stage. Secondary pairs of pioneer neurons are known to occur in the grasshopper's antenna (Bate, 1976*a*) and leg (Keshishian, 1980*b*), where each separate pair establishes a different major branch of the peripheral nerve. A second branch is added to the cercal sensory nerve at about 50%, suggesting that the cercus may also possess an additional pair of later-arising pioneers.

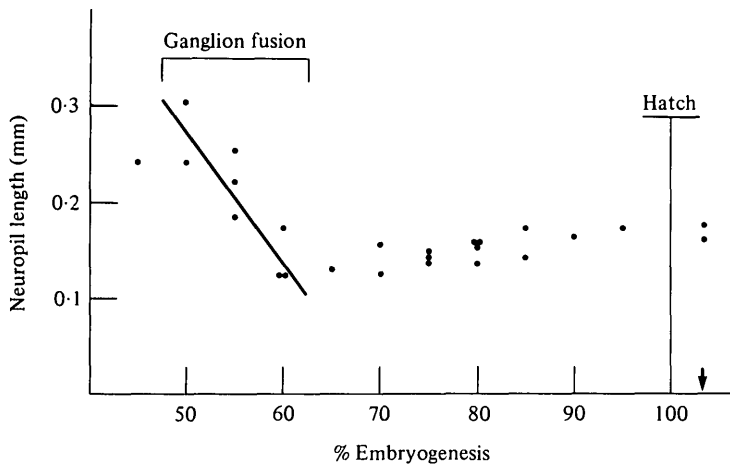


Fig. 3. Terminal ganglion fusion is accompanied by a shortening of the longitudinal tracts connecting the neuropil of segments A_8 – A_{11} . A line ($r = 0.88$) has been fitted to the data points taken during the period of fusion. Arrow indicates one day posthatching.

Central nervous system

At the time the peripheral pioneer axons arrive the embryonic CNS consists of segmental rudiments which have not yet assumed their mature shape or configuration. The principal neuronal precursor cells, the neuroblasts (Bate, 1976*b*), are still present and dividing at this stage. These cells lie against the ventral body wall epidermis and can be distinguished from surrounding epithelial cells by their large size ($25\ \mu\text{m}$ dia.), finely textured chromatin, and high incidence of mitotic figures. Differentiating neurons lie dorsal to the neuroblasts, and axons grow in bundles over the most dorsal surface of the rudimentary CNS (Fig. 1*B*). These first central fibres arise at progressively later stages in the more posterior segments. They are first seen in the thorax at 30%, but not until 40% in the terminal abdomen. The cercal pioneer axons grow into the terminal ganglion rudiment at roughly the same time, and therefore contribute to the central neuropil at an early stage in its formation.

The anatomy of the embryonic neuropil was reconstructed from serially sectioned tissue stained by the Rowell silver technique (Fig. 2). Each segment has an initial pair of transverse commissures that are joined into a chain by a pair of longitudinal tracts which run the nerve cord's length. (The anterior segmental commissure consists of two closely apposed fibre tracts of distinct embryonic origin, so there are in fact three separate primary commissural tracts per segment – C. S. Goodman & C. M. Bate, personal communication). The paired commissures and their affiliated cell bodies transform into segmental ganglia when the CNS detaches from ventral body wall at 50–60%, while the intervening longitudinal tracts are drawn out to form the interganglionic con-

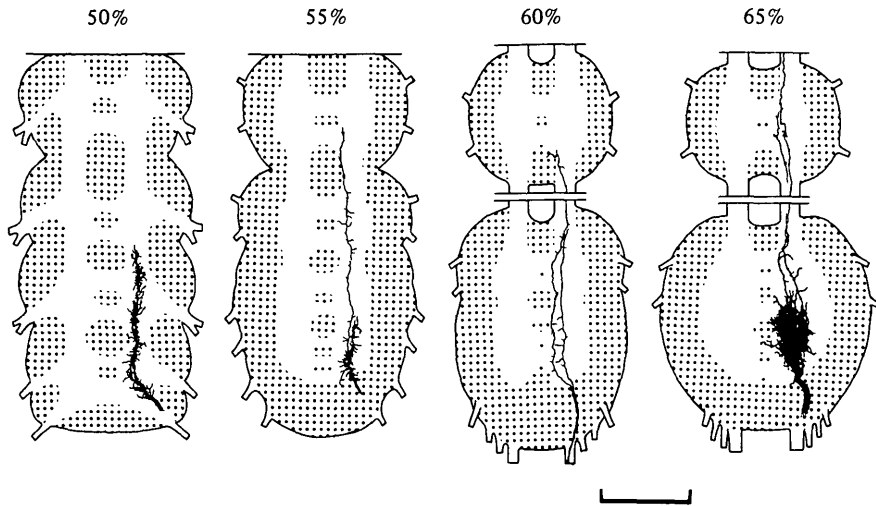


Fig. 4. Cercal pioneer axons projecting into the fusing terminal ganglion at a series of embryonic stages. The pioneers grow anteriorly along the ipsilateral longitudinal tract, progressing further into the neuropil as embryogenesis proceeds. Short lateral filopodia line the sides of the axon shaft at 50%, but these processes later disappear. The first sensory axons enter the terminal ganglion at 65% and form two clumps of densely interwoven arbor. Camera-lucida tracings of silver-intensified cobalt fills. Scale 100 μm .

nectives. However, the rudiments of the eighth to eleventh embryonic abdominal segments (A_8 – A_{11}) do not separate into individual ganglia, but rather fuse into a single terminal ganglion with a common neuropil (Fig. 2; see also Roonwal, 1937, and Panov, 1964). It is this fused ganglion which receives the cercal nerve.

Two distinct morphological processes account for the fusion of the terminal ganglion neuropil. (i) The continual deposition and elaboration of neuronal processes swells the pre-existing fibre tracts. When adjacent commissures meet and fuse, they displace the intervening cell bodies to the cortex of the ganglion. (ii) While most of the nerve cord is elongating, the commissures which will contribute to the terminal ganglion are being drawn together from their original locations by a shortening of the longitudinal tracts which connect them (Fig. 3). Connective shortening is a common feature of ganglion fusion in insects, and has been correlated with a looping or buckling of the constituent axons (Pipa & Woolever, 1964). However, cobalt fills of individual cercal pioneer axons which are growing through the shortening region of the grasshopper embryo's longitudinal tract show no evidence of looping (Fig. 4).

Central projections of the peripheral pioneer axons

Cobalt filling the cercal nerve at various stages of development shows that there are two distinct classes of cercal afferents which reach the CNS at different times in embryonic life (Fig. 4). (i) The first class consists of a small number of

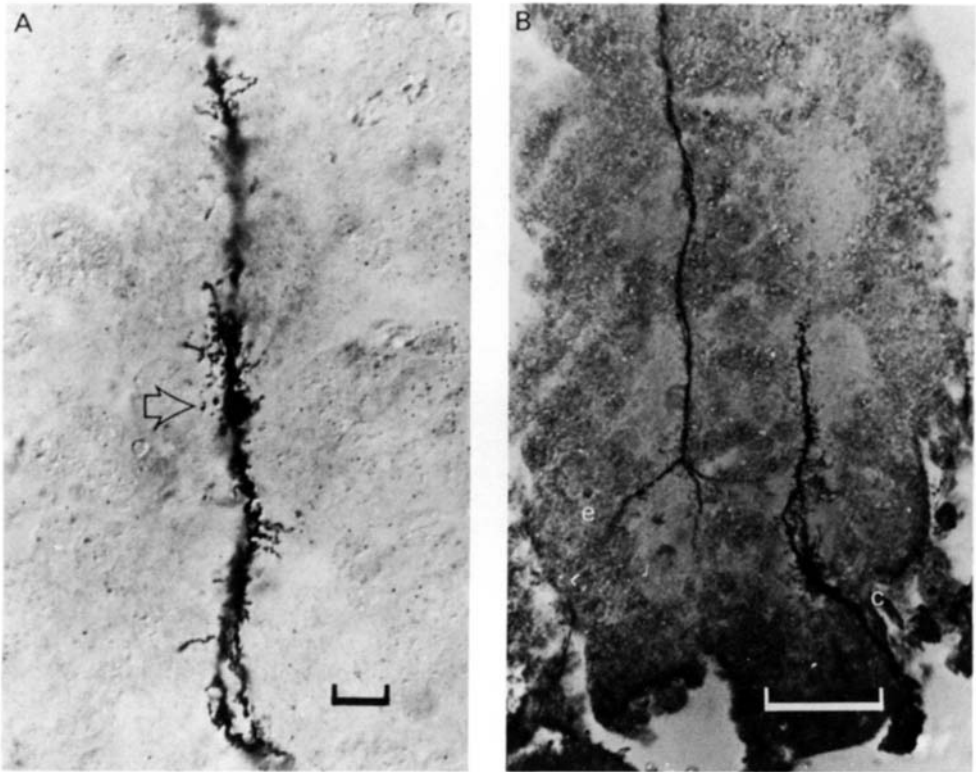


Fig. 5. Central projections of the peripheral pioneer axons stained by silver-intensified cobalt fills. Photomicrographs taken from $25\ \mu\text{m}$ horizontal sections of the embryonic terminal ganglion in the midst of its fusion. (A) The two cercal pioneer axons follow an exclusively anterior course, and do not branch within the CNS. The growth cone (arrow) of one pioneer axon grows along the shaft of the other, and thus follows the same pathway with a slight delay. Filopodia emanate from the leading edge of the growth cone and from the sides of both axon shafts. (50% embryo) Scale $10\ \mu\text{m}$. (B) The epiproct nerve (e), which enters the terminal ganglion one segment anterior to the cercal nerve (c), also contains early-differentiating afferent axons that grow anteriorly along the ipsilateral longitudinal tract. However, some of these presumed epiproct pioneer axons branch where they enter the neuropil and send collaterals posteriorly and to the contralateral side of the ganglion (55% embryo). Scale $50\ \mu\text{m}$.

long, unbranched axons which follow a common pathway through the ganglion. These are the only type of cercal afferent stained by cobalt fills prior to 65%, and they are believed to be the central projections of the pioneer neuron pair that was seen in the periphery. The fact that both of the earliest successful fills, performed on 50% embryos, stained only a single pair of axons is consistent with this idea (Fig. 5A). However, the initial pair of cercal pioneer neurons do not seem to be the only source of these long, unbranched axons, because some of the successful cobalt fills performed at 55–60% (three out of eight) stained three different cercal afferents of this type. The additional long, unbranched axon(s)



Fig. 6. Growth cones of the cercal pioneer axons, traced by camera lucida from silver-intensified cobalt fills. The growth cone is an ellipsoidal swelling of the axon shaft with a long terminal filament projecting along the path the axon is taking, in this case towards the top of the page. Variable numbers of filopodia radiate from the leading edge of the growth cone and from the sides of the filament (55–60% embryos). Scale 10 μm .

stained in these fills may derive from secondary pioneer neurons which establish other peripheral branches of the cercal nerve. (ii) The second class of cercal afferents begin to enter the terminal ganglion in large numbers and form densely interwoven clumps of arborization at 65% (Fig. 4). These axons derive from the epidermal sensory neurons and their development will be considered at length in the following paper (Shankland, 1981). Cobalt fills of the cercal nerve did not stain efferent neurons with central somata at any stage.

The axons of the pioneer neurons terminate in obvious growth cones within the CNS (Fig. 5A, 6). These growth cones would appear to be actively engaged in axon elongation at the time they were stained because the fibres project further into the CNS with each succeeding stage (Fig. 4). The typical growth cone is a 2–5 μm ellipsoidal swelling of the 0.5–1.0 μm axon shaft. The long axis of the growth cone points along the axon's pathway, and fine (0.2–0.3 μm dia.), largely unbranched filopodia radiate in various directions from its leading edge. Each growth cone also has a single long (25–50 μm) terminal filament which extends in advance of the axon and has filopodia emanating from its sides. Although the diameter of this terminal filament could not be distinguished from that of a filopodium with silvered cobalt fills, the staining of other axonal growth cones with the fluorescent dye Lucifer Yellow has shown that the filament is about twice as thick (M. Shankland & C. S. Goodman, unpublished results).

The growing cercal pioneers also have lateral filopodia emanating from the sides of the axon shaft in the growth cone's wake (Fig. 5A). These processes are short (5–20 μm), unbranched, and extend perpendicular to the axon's path. They are found throughout the neuropil, but not within the cercal nerve. The pioneer axon loses these lateral filopodia as embryogenesis proceeds (Fig. 4).

There are an average of 55 filopodia/100 μm of axon shaft ($n = 4$ axons) at 50%; 36 filopodia/100 μm ($n = 8$) at 55%; and only 6 filopodia/100 μm ($n = 4$) at 60% of embryonic life. The lateral filopodia are uniformly distributed along the axons' central extent throughout this time, so their loss is independent of proximity to the growth cone.

Cobalt fills of the cercal pioneers confirm the *in vivo* Nomarski observations of the axons' pathway through the CNS. The pioneers enter the posterolateral corner of ganglion rudiment A_{11} , then turn toward the anterior to join the axons of the ipsilateral longitudinal tract. They follow an exclusively anterior course along the medial edge of this tract, and pass through the four segmental rudiments of the terminal ganglion (A_{11} – A_8) into more anterior ganglia A_7 and A_6 . Although the two axons follow the same basic path, their growth cones do not advance in unison. In no instance were two pioneer axon growth cones found to be in contact, although growth cones often appeared to be following along the axon shaft of the leading pioneer (Fig. 5A).

The cercal pioneer axons do not form secondary growth cones or branches prior to 65%, although they grow through several hundred microns of neuropil during this time. It is not known whether they branch at a subsequent stage because the sensory axons grow into the ganglion and obscure the pioneers in later cobalt fills.

Cobalt fills of the neighbouring epiproct nerve at 55–60% of embryogenesis stain long, unbranched afferent axons (Fig. 5B) which are presumed to be peripheral pioneer axons for that nerve. They are few in number (a maximum of four were stained by a single fill), have ellipsoidal growth cones and lateral filopodia, and turn anteriorly to follow the ipsilateral longitudinal tract. However, some of these putative epiproct pioneer axons differ from the cercal pioneers in that they branch where they enter the neuropil, sending secondary growth cones posteriorly in the longitudinal tract or across the ganglion in the anterior commissure of A_{10} (Fig. 5B). These collaterals, like the long anterior axon, show no signs of subsidiary arborization.

Do cercal pioneer neurons persist into postembryonic life?

Although the fate of the cercal pioneer neurons could not be followed within the CNS after 65%, cobalt fills of the neurons lying within the cercus suggest that the pioneers persist into postembryonic life. There are a pair of afferent neurons in the cercus of the first instar grasshopper which have a striking resemblance to the pioneers seen during embryogenesis (Fig. 7, compare to Fig. 1E). Unlike the majority of cercal afferents, which are sensory neurons with somata embedded in the epidermis, these two cells lie within the cercal lumen at the head of the cercal nerve. At least one of these neurons has an unbranched apical dendrite which extends into the distal tip of the lumen and comes to an abrupt termination without crossing the epidermis or making contact with a cuticular sensillum (Fig. 7B). Dendrites of this sort have been

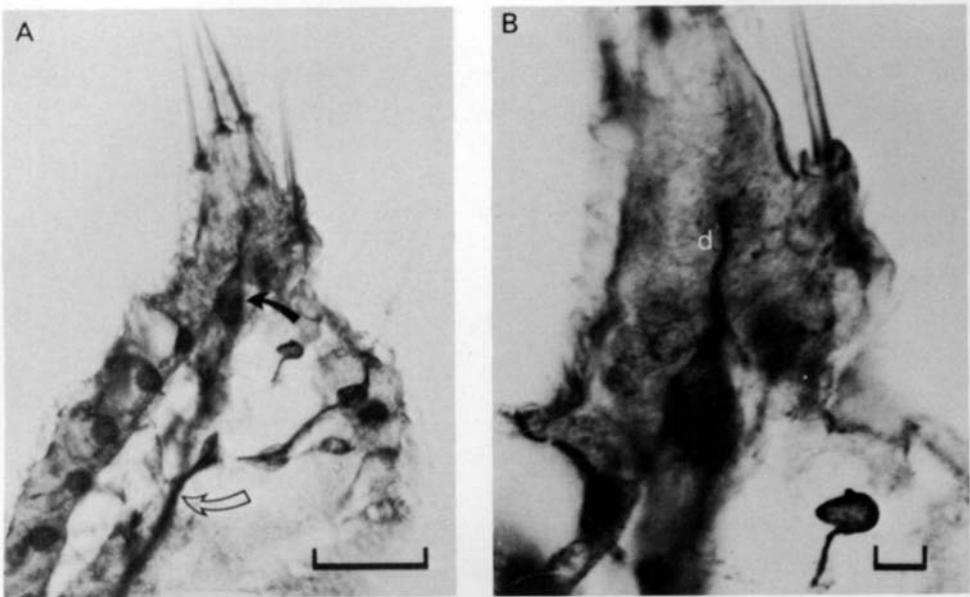


Fig. 7. Pioneer and sensory neurons in the cercus of a first instar grasshopper. Photomicrograph of a $40\ \mu\text{m}$ section from a silver-intensified cobalt fill. (A) A pair of afferent neurons (solid arrow) lying within the lumen at the head of cercal nerve (hollow arrow) are believed to be the cercal pioneer neurons surviving into post-embryonic life. The other cercal afferents seen in this section are all sensory neurons with their somata embedded in the epidermis. Scale $50\ \mu\text{m}$. (B) Same specimen, higher magnification. One of these cells has an unbranched apical dendrite (*d*) which terminates within this section without crossing the epidermis or contacting a cuticular sensillum. Scale $10\ \mu\text{m}$.

previously described on embryonic pioneer neurons with both electron microscopy (Edwards & Chen, 1979) and intracellular dye fills (Keshishian, 1980*a*). Such similarities suggest, but do not prove, that this pair of cells is in fact the cercal pioneer neurons surviving after embryogenesis ends.

DISCUSSION

The cercal nerve of the grasshopper is established by an identifiable pair of pioneer neurons at 40% of embryonic life. These two cells are the first neurons to differentiate in the cercus, and their axons lay down the pathway of the cercal sensory nerve by growing from the cercus to the rudimentary CNS. Cobalt fills show that there are no efferent axons which project into the cercal bud, so it would appear that the cercal pioneer axons are the first fibres to cross this gap. Glial cells enwrap these axons soon after they appear, and large numbers of sensory axons follow this nerve to the CNS at a later stage.

The cercal pioneer neurons described in this study are quite similar to the peripheral pioneer neurons which establish other insect nerves. The pioneering

axons of the antennal (Bate, 1976*a*; Toroian-Raymond & Bentley, 1980) and leg (Bate, 1976*a*; Keshishian, 1980*a*) nerves also derive from discrete pairs of sub-epidermal neurons which arise at the distal tip of the embryonic appendage. Keshishian (1980*a*) has shown that the pioneer neuron pair which lays the foundation for nerve 5b1 in the metathoracic leg lies between the epidermis and an inner mesodermal epithelium, but the cercus contains no such mesodermal layer and the cercal pioneers are directly exposed to the lumen. The afferent neurons which establish the cercal nerve of the cricket embryo also have sub-epidermal somata (Edwards & Chen, 1979), although none of these cells have been individually identified as yet. The similarities between these various studies suggest that the peripheral pioneer neurons which establish afferent nerves in the different appendages of the insect embryo are segmentally homologous members of a single discrete class of cells.

The peripheral pioneer neurons are distinguished from the majority of other insect afferents by certain anatomical peculiarities, as well as their early differentiation. The pioneer neuron is a bipolar nerve cell with a single unbranched apical dendrite which is not associated with a cuticular sensillum (Edwards & Chen, 1979; Keshishian, 1980*a*). This dendrite was not seen here in the Nomarski observations, but could be recognized in the cobalt fills of the cercal pioneer cell bodies during postembryonic life. Bipolar morphology, as well as the ciliary ultrastructure of the dendrite (Edwards & Chen, 1979), distinguishes the pioneer from the multipolar type-2 insect sensory neurons which innervate receptor muscles and line the bodywall (Zawarzin, 1912). Such characteristics are typical of the type-1 sensory neurons which innervate sensory hairs and scolopidial organs, but all previously reported type-1 afferents have a cuticular transducing element associated with their dendrites. Hence, the peripheral pioneer neuron is not typical of either of the two generally accepted classes of insect sensory afferents (Finlayson, 1968).

The cercal pioneer neurons also behave quite differently from the sensory neurons of the cercal epidermis within the CNS. The cercal pioneer axons do not arborize for over 20% of embryonic development, despite growing through several hundred microns of neuropil. The putative pioneer axons of the epiproct nerve form collaterals where they first enter the CNS, but likewise project through several ganglia without subsidiary arborization. In contrast, the axons which derive from epidermal sensory neurons begin to branch repeatedly within 5% of embryonic development after arriving in the CNS (Shankland, 1981). The pioneer axons may also branch at some later, unobserved stage, but the prolonged absence of arborization distinguished the pioneers from epidermal sensory cells.

Although the peripheral pioneers are afferent neurons, it is not known whether they serve any sensory function. The lack of a cuticular sensillum and the failure to form an embryonic arborization argue against such a sensory role, but there is no evidence pertaining to the pioneer neuron's capacity to electric-

ally conduct or synaptically distribute sensory information during the same stages that sensory neurons are known to exhibit these characteristics. A further note of caution arises from certain parallels between the peripheral pioneer neurons and a class of early-differentiating central neurons, the progeny of the midline precursors (MPs), whose long, unbranched axons establish some of the first central fibre tracts (Bate & Grunewald, 1981; Goodman, Bate & Spitzer, 1981). Many of the MP progeny remain unbranched from the beginning of axonogenesis (at 30% in the metathorax) until they die (50–60%), and fail to develop the membrane properties necessary for action potential initiation or propagation (Bate, Goodman & Spitzer, 1981). But one of the MP progeny, the H cell, undergoes a secondary morphological and physiological transformation, becoming a highly arborized and electrically active neuron which survives into later life (Goodman *et al.* 1981). It may be that the peripheral pioneers which were seen in the first instar cercus have also undertaken a delayed arborization and possess typical sensory axon branching patterns.

The cercal pioneer neurons arise later in embryogenesis than their counterparts in the antenna or leg. Keshishian (1980*a*) reports that the metathoracic leg pioneers undergo axonogenesis at 30%, and the antennal pioneers appear soon thereafter (Bate, 1976*a*). However, the first pioneer neuron pair was not seen in the cercus until 40% of embryonic life. There is an equivalent 10% lag between thorax and terminal abdomen in both the onset of neuroblast divisions (Bate, 1976*b*) and the appearance of the first central fibre tracts, so it would appear that pioneer neuron differentiation is one of many events in nervous system development which exhibit the anteroposterior gradient of segmental maturation common to insect embryogenesis (Anderson, 1972). The cricket's cercal nerve pioneer neurons differentiate at an even later stage in embryogenesis than those of the grasshopper. The cricket's pioneers are first seen after the completion of katatrepsis, and coincident with the beginning of cercal bud elongation (Edwards & Chen, 1979), while the pioneer neurons of the grasshopper cercus are found prior to the initiation of either event.

The lateral filopodia seen on the immature pioneer axon are ephemeral structures which normally accompany the growth of neuronal processes in the grasshopper embryo (Goodman & Spitzer, 1979; Keshishian, 1980*a*; Shankland, 1981) as well as the mammalian brain (Morest, 1968, 1969). It is not known whether these filopodia are left behind by the growth cone or actually sprout from the sides of the axon shaft, but the lack of a correlation between their distribution and the location of the growth cone suggests that they have an independent existence and actually mediate some interaction of the immature axon with its surrounding tissues. Morest (1968, 1969) proposes that lateral filopodia are involved with incipient branch formation, but this is certainly not the case for the branchless pioneers. One alternative possibility is that the lateral filopodia bind together newly associated fibre bundles by increasing the cell surface area available for adhesion. The tracts of the rudimentary neuropil are

not yet supported by glia at this stage (Edwards & Chen, 1979), so enhanced cohesion between bundled axons would help to withstand potentially disruptive morphogenetic forces (e.g. tract shortening) and the invasive action of passing growth cones.

Bate (1976a) has suggested that glia might serve as stepping stones which mark the pioneer's centripetal route, but little is actually known about the parameters which guide the pioneer axon in either the periphery or the CNS. The pathway chosen by the growing axon is one source of information about such guidance cues. The pioneer axons of both the cercal and epiproct nerves make abrupt turns where they enter the ganglion, forsaking their original trajectories to follow the axons of the longitudinal tract. Hence, the pioneers respond to their first encounter with other neural tissue by joining an already established axon bundle. The sensory axons, which follow the pioneers from the periphery to the CNS, exhibit this same response when confronted with a pre-existing neural path (Wigglesworth, 1953).

The morphology of the pioneer axon's growth cone also has implications for the nature of axonal guidance. The terminal filament arising from this growth cone always points along the longitudinal tract in the axon's advance, and therefore marks the path the axon is taking. This suggests that the cellular interactions which guide the axon occur on this filament, while the bulbous growth cone is simply a site for the addition of new materials to the growing tip. The axonal growth cones of sensory neurons (Shankland, 1981) and interneurons (M. Shankland & C. S. Goodman, unpublished) often exhibit this same morphology when growing along established tracts, but typically do not have terminal filaments at other points along their path, particularly where they branch.

The pioneer axons (or their nerve) serve in turn as guidance cues for other axons. When the sensory axons grow from the epidermis into the lumen, they accumulate around the pioneer nerve and follow it to the CNS (Bate, 1978; Edwards & Chen, 1979). Since the nerve has separated from the epidermis over much of its length before the sensory neurons even appear, it is unlikely that their axons are following the same cues that originally guided the pioneers along the bodywall. Furthermore, ablation experiments have shown that sensory axons require a pre-existing nerve to aggregate in their normal fashion (Edwards *et al.* 1981). Thus, it would seem that the sensory axons do use the pioneers as a pathway through the periphery, and may continue to use them for guidance within the CNS as well (Shankland, 1981). But the fact that the sensory axons *do* follow the pioneers does not mean that they *must* follow them to arrive at their central destinations. It is possible that the sensory axons have alternative means of reaching their target ganglia that are not utilized during normal development. This raises the fundamental, and as yet unsolved, question about the developmental and evolutionary role of the pioneer neuron. Are the pioneers nothing more than the first type of sensory afferent to differentiate, with no special pathfinding capacity or opportunity? Or have they evolved to serve a

unique function in development by establishing a fundamental nerve route which is a necessary prerequisite for the accurate connexion of sensory afferents into the CNS?

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