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The early development of motor axon pathways in the locust embryo: the establishment of the segmental nerves in the thoracic ganglia

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Summary

This study has identified the first five motor neurones to send axons out of the segmental nerves in the thoracic ganglia of the locust and has traced the pathways followed by these axons up to their divergence into the ganglionic nerve roots. These motor neurones send out axons in a stereotyped sequence over a short period, corresponding to 2 % of embryonic development. Motor axons initially grow dorsally to contact the dorsal basal lamina and then posteriolaterally in a parallel array just beneath this membrane. At the edge of the CNS the axons diverge into either of two pathways: an anterior pathway, corresponding to nerve root 3 which is pioneered by the first motor axon to leave the CNS; and a posterior pathway, corresponding to nerve root 5, which is pioneered by the second motor axon. The first motor axon appears to grow circumferentially around the segmental border between the body wall and the base of the coxa, while the second is closely associated with the filopodia or axons of the afferent peripheral pioneer neurones. The later motor axons reliably follow the pathways pioneered by these first two axons. A small number of molecular markers would be sufficient to generate the observed patterns of axon growth by these early motor neurones and some of these same cues may be used to guide afferent axons into the CNS.

Key words: insect embryo, axon guidance, motor neurone, locust, neural development.

Introduction

Several recent studies in the locust embryo have described the spatiotemporal patterns of axon growth from identified interneurones (e.g. Bastiani *et al.* 1986) and sensory neurones (e.g. Heathcote, 1981; Caudy & Bentley, 1986*b*) during normal development. These studies have made a major contribution to our understanding of how axons manage to locate their synaptic targets.

Although a number of identified motor neurones have been located in the locust embryo (Whitington & Siefert, 1981; Ball *et al.* 1985; Kotrla & Goodman, 1984), little is known about the patterns of axon growth from this class of neurones. Such information would be valuable for at least two reasons: (1) to establish whether common mechanisms for axon guidance operate in all three major classes of neurones, and (2) since the synaptic targets of motor neurones are more easily identified and are more accessible than those of either interneurones or sensory neurones, the later stages of axon growth towards and over the target are more readily studied in motor neurones than in the other two classes of neurones.

As a first step towards discovering the mechanisms for guidance of motor axons to their targets, I have concentrated on the first motor axons to leave the central nervous system (CNS). These pioneering motor axons establish the initial grid which later-outgrowing motor axons follow. In this paper I describe the pathways taken by these axons as they make their way out of the CNS via the segmental nerves in the thoracic ganglia.

Materials and methods

Embryos were obtained from a laboratory culture of locusts *Locusta migratoria* maintained at the University of Melbourne and the University of New England.

For intracellular dye injection, embryos were dissected from the egg case under embryonic locust Ringer (Raper *et al.* 1983) and staged from external morphological features (Bentley *et al.* 1979). They were then positioned dorsal side up in a well cut into a Sylgard-covered microscope slide (Raper *et al.* 1983) and examined in a Zeiss Standard microscope using a Leitz ×100 water immersion objective and Nomarski optics. Motor axons on the dorsal surface of the developing ganglion were visualized, penetrated with 30–60 MΩ microelectrodes filled with 5 % lucifer yellow (LY) and iontophoretically injected with dye using a 0·2 nA hyperpolarizing D.C. current for several seconds.

The embryo was then fixed in 4 % paraformaldehyde in

Millonig's buffer for 30 min, washed in phosphate-buffered saline (PBS), and incubated for 2 h in anti-LY antibody diluted 1:500 in PBS with 0·1 % Triton X-100 and 0·25 % bovine serum albumin (BSA). The anti-LY antibody, which was raised in rabbit, was a kind gift from Dr Hajime Fujisawa of Kyoto University. After washing in PBS, the embryo was incubated for 2 h in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody, diluted 1:250 in PBS with Triton X-100 and BSA. A wash in PBS was followed by 20 min incubation in 0·5 % diamino-benzidine in PBS. Hydrogen peroxide was added to a final concentration of 0·006 % and the reaction allowed to proceed until the injected neurone became darkly stained. Finally the embryo was washed in PBS and cleared and mounted in 100 % glycerol.

To stain all peripheral axons, embryos were treated with anti-HRP antibody (Jan & Jan, 1982). The protocol used was identical to the anti-LY procedure except that the primary antibody was used at a dilution of 1:250 for 6–12 h and the secondary antibody at 1:500 for 12 h.

Stained neurones were drawn with the aid of a camera lucida on a Zeiss Photomicroscope with Nomarski optics, using a ×100 Plan oil immersion objective.

Peripheral pioneer neurones were named using the terminology of Caudy & Bentley (1986*a*).

Results

General comments

The following description of axon growth from motor neurones is based upon a combination of LY fills to determine the morphology of single motor neurones and anti-HRP staining to reveal the entire population of motor and sensory axons. While most observations were made on the metathoracic ganglion, some data are included from the pro- and mesothoracic ganglia, whose pattern of development is very similar to that seen in the metathorax.

Observation of embryos older than 33 % reveals a row of axons in the anteriolateral region of the ganglion, lying in one plane just beneath the basal lamina which covers the dorsal side of the neuroepithelium (Fig. 1). By repeated filling of axons across this row in embryos between 30 and 35 % of development ($n > 70$), I have identified the first five motor axons to leave the CNS via this route. Fig. 1 shows the positions of the somata of these motor neurones. Each lies in a particular position in the dorsal–ventral axis; either just beneath the basal lamina (d), just above the ventral layer of neuroblasts (v), or midway in between (m). The axons grow out in a stereotyped sequence (indicated by the numbers on the somata) and each is found in a characteristic position in the lateral row (see below).

Early stages of axon growth

Since motor neurones were stained by injection of LY into the axon just beneath the basal lamina, the very earliest stages of axonogenesis were not observed directly in this study. However, motor neurone growth cones which have just contacted the basal lamina generally lie directly above the soma (e.g. Fig. 2, 31 %), suggesting that the axon grows dorsally from the soma to the nearest point on the basal lamina.

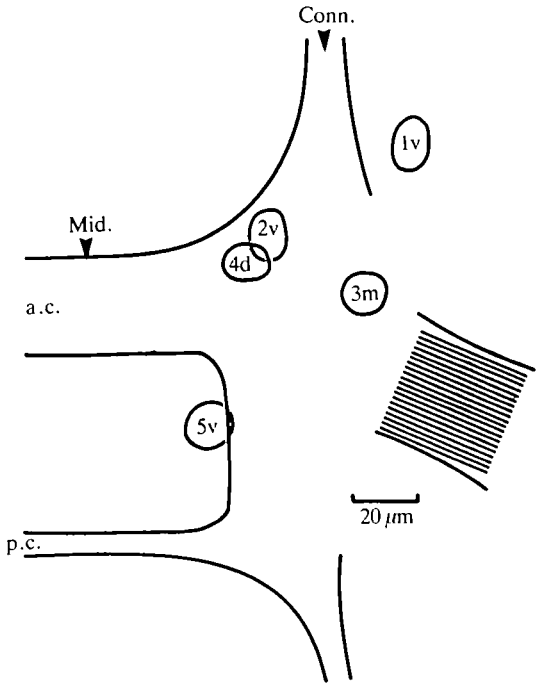

Fig. 1. Diagram of a methathoracic ganglion at 34 % of development showing the position of the somata of the first 5 motor neurones to send axons out of the segmental nerves. The numbers on the somata indicate the order in which the axons arrive at the basal lamina, while the letters signify the positions of the somata in the dorsoventral axis; v, ventral, d, dorsal; m, midway between dorsal and ventral. The axons lie on the basal lamina within the region indicated by the parallel lines. a.c., anterior commissure; p.c., posterior commissure; mid., midline; conn., connective.

At later stages of development the soma usually lies some distance removed from the point of contact of the axon with the basal lamina (e.g. Fig. 2, 33 %, 34 %). This displacement is probably a manifestation of a general passive movement of neurone somata caused by the continued generation of neurones.

After contacting the basal lamina all motor axons initially show a similar pattern of growth: they grow in a straight line in a posteriolateral direction, relative to the point at which the axon contacts the basal lamina, directly beneath the basal lamina. (Note that in the mean time the motor neurone somata have usually been displaced and no longer lie beneath the original point of contact of the motor axon with the basal lamina). An extensive array of filopodia, most of which appear to be in contact with the basal lamina, radiates from the axon. During this phase of their growth the axons rarely cross each other: rather they grow parallel to earlier motor axons. Branching of the axon is often observed in this region (e.g. Fig. 3, 33 % and 34–35 %).

Up to this point the first five motor axons have been treated as a group since they all show the same pattern of growth until they reach the edge of the CNS (approximately 100 μm lateral to the midline in a 33 % embryo and indicated by a dashed line in Figs 2–4). Thereafter, however, the behaviour of motor axons

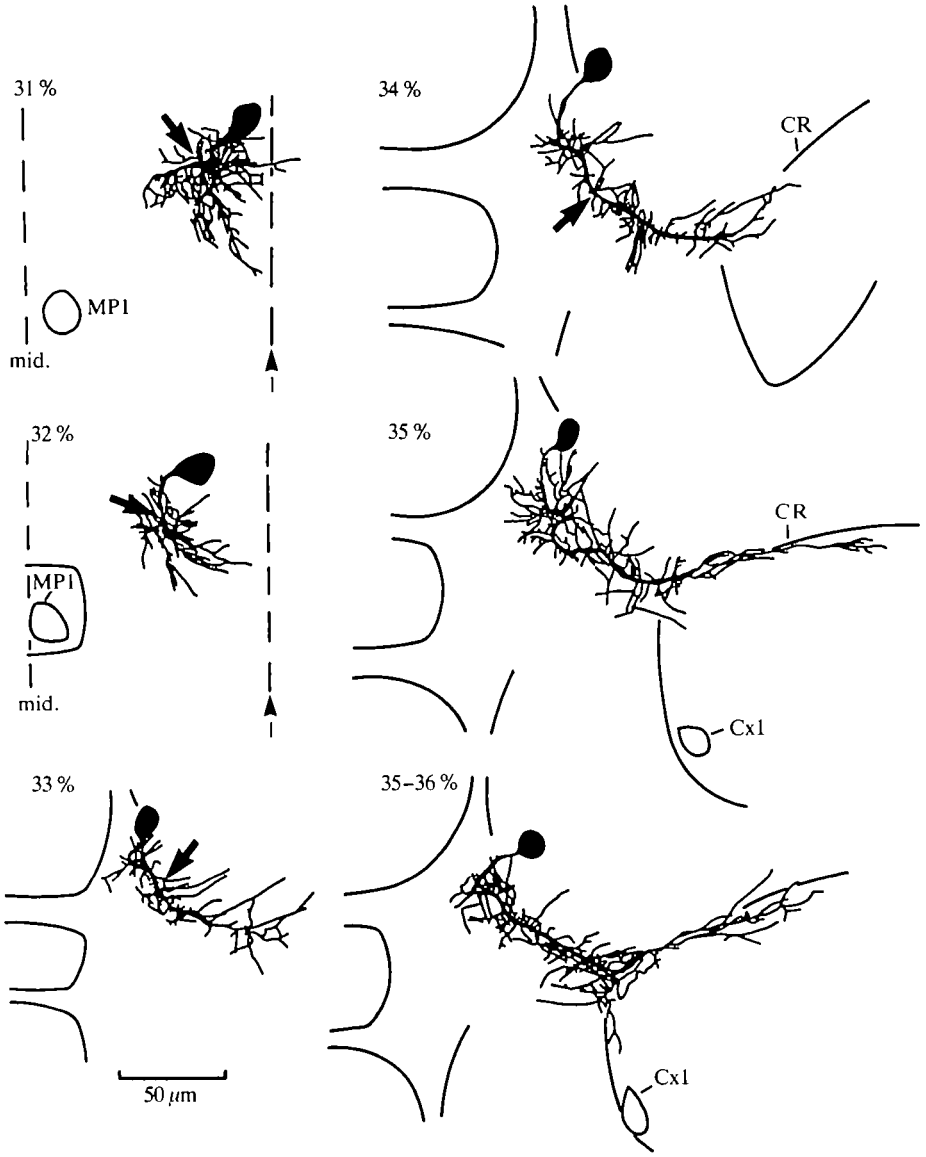

Fig. 2. Camera-lucida drawings of motor neurone 1 between 31 and 36 % of development, drawn from LY fills. The arrows indicates the point where the axon contacts the basal lamina. CR, coxal rim; Cx1, cell body of Cx1 neurone; MP1, cell body of MP1 neurone; l, lateral edge of neuroepithelium.

begins to differ and hence they are described individually.

Motor axon 1

Motor axon 1 (Fig. 2) is the first to reach the edge of the CNS, arriving here at 33 % of development. The pathway taken by this axon further in the periphery, together with the position of the soma, identifies this neurone as slow extensor tibiae (SETi), one of the four motor neurones that innervate the main jumping muscle in the metathoracic leg.

At this stage axon 1 is the most anterior in a group of 4–5 motor axons which have contacted the basal lamina. The growth cone is expansive. Filopodia are present along the length of the axon but are especially abundant at the tip, where they radiate out from the growth cone in a planar array just beneath the basal lamina. They are up to 65 μm long.

By 34 % motor axon 1 has grown ventrally along the basal lamina to join the epithelium on the ventral side of the body. At 35 % a prominent group of long (up to 85 μm) filopodia is directed anteriorly and appears to adhere to the epithelium on the anterior rim of the base of the coxa. A cluster of shorter filopodia often extends posteriorly from the growth cone (Fig. 2, 35–36 %).

By 35–36 % motor axon 1 has turned anteriorly, following the long filopodia along the anterior coxal rim (Fig. 2, 35–36 %). No other axons, either motor or sensory, are present in this immediate region. Motor axon 1 has been described at this stage in an earlier report by Keshishian & Bentley, (1983).

The pathway pioneered by motor axon 1 corresponds to nerve root 3. The later development of this motor neurone will be described in a subsequent paper.

Motor axon 2

Motor axon 2 follows closely behind motor axon 1 in time, reaching the edge of the CNS by 33 to 34 % of development (Fig. 3). At this stage motor axon 2 lies in the middle of a row of approx. 6 axons on the basal

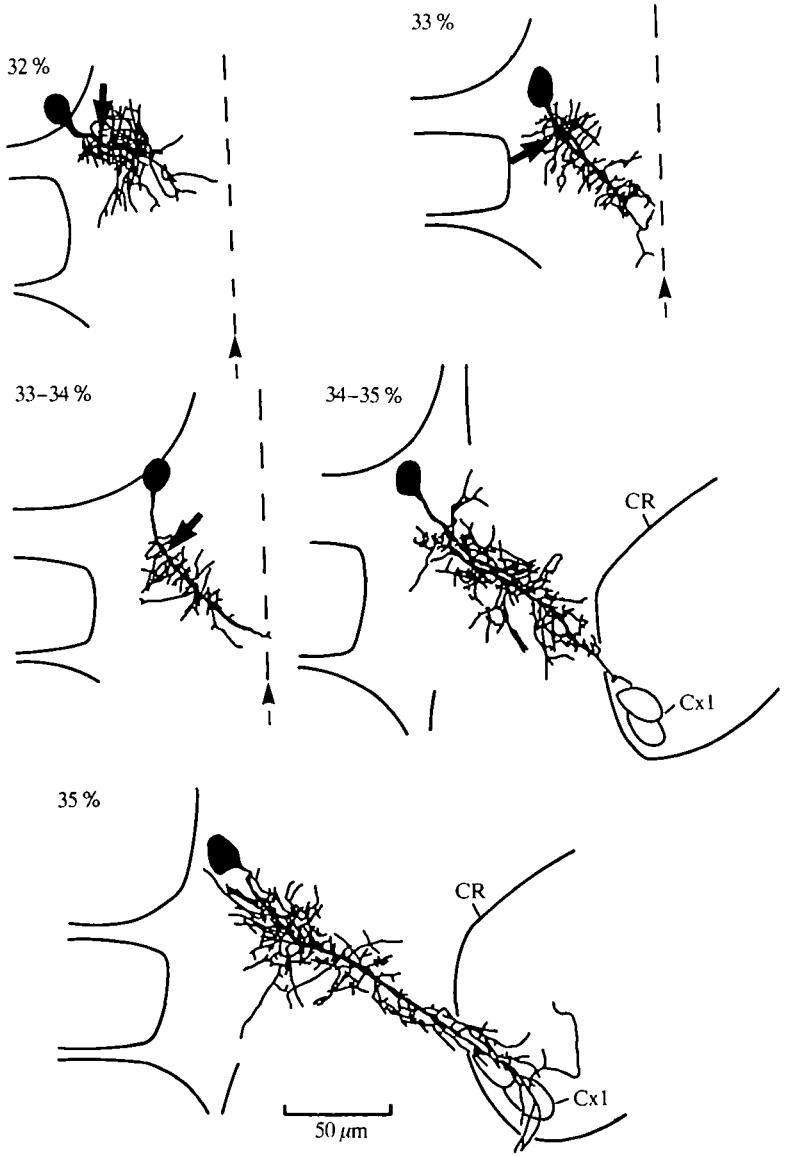

Fig. 3. Camera-lucida drawings of motor neurone 2 between 32 and 35 % of development, drawn from LY fills. The arrow indicates the point where the axon contacts the basal lamina.

lamina. Filopodia are abundant along the length of the axon. These point in virtually all directions in the plane immediately beneath the basal lamina. Some filopodia contact the path taken by motor axon 1.

By 34–35 % motor axon 2 has met the ventral epithelium and has advanced further along its prior trajectory which begins to take it into the coxa. Some long filopodia extend along this path. Anti-HRP stained preparations reveal that these filopodia are closely associated with the filopodia of peripheral pioneer neurones (neurones Cx1 or Ti1, previously identified by Keshishian & Bentley, 1983 and Ho & Goodman, 1982), whose axons are growing in towards the CNS. In the metathorax, filopodia of motor axon 2 first contact the filopodia of the Ti1 neurones whereas in the pro- and mesothorax the Cx1 filopodia are generally the first contacted. The point where motor axon 2 and the peripheral pioneer axons meet varies, due to variability in the timing of ingrowth of the peripheral pioneer axons: in some embryos these axons meet at the edge of the CNS, while in others they meet close to the Cx1 somata. The path pioneered by motor axon 2 and the Ti1/Cx1 axons corresponds to nerve root 5.

Although motor axon 2 has filopodial access to the nerve root 3 pathway, it does not grow down this route, choosing instead the nerve root 5 path. Conversely, motor axon 1 always chooses nerve root 3 over root 5 even though it has access to the latter pathway (as indicated by the presence of filopodia).

By 35–36 % the growth cone of motor axon 2 is at the Cx1 somata.

Motor axon 3

Motor axon 3 closely follows motor axon 2, arriving at the edge of the CNS between 33 and 34 % of development (Fig. 4A). At this stage this axon lies in the middle of the motor axon array. By 35 to 36 % motor axon 3 has grown into nerve root 5, closely associated with motor axon 2, and its growth cone approaches the Cx1 somata (Fig. 4B).

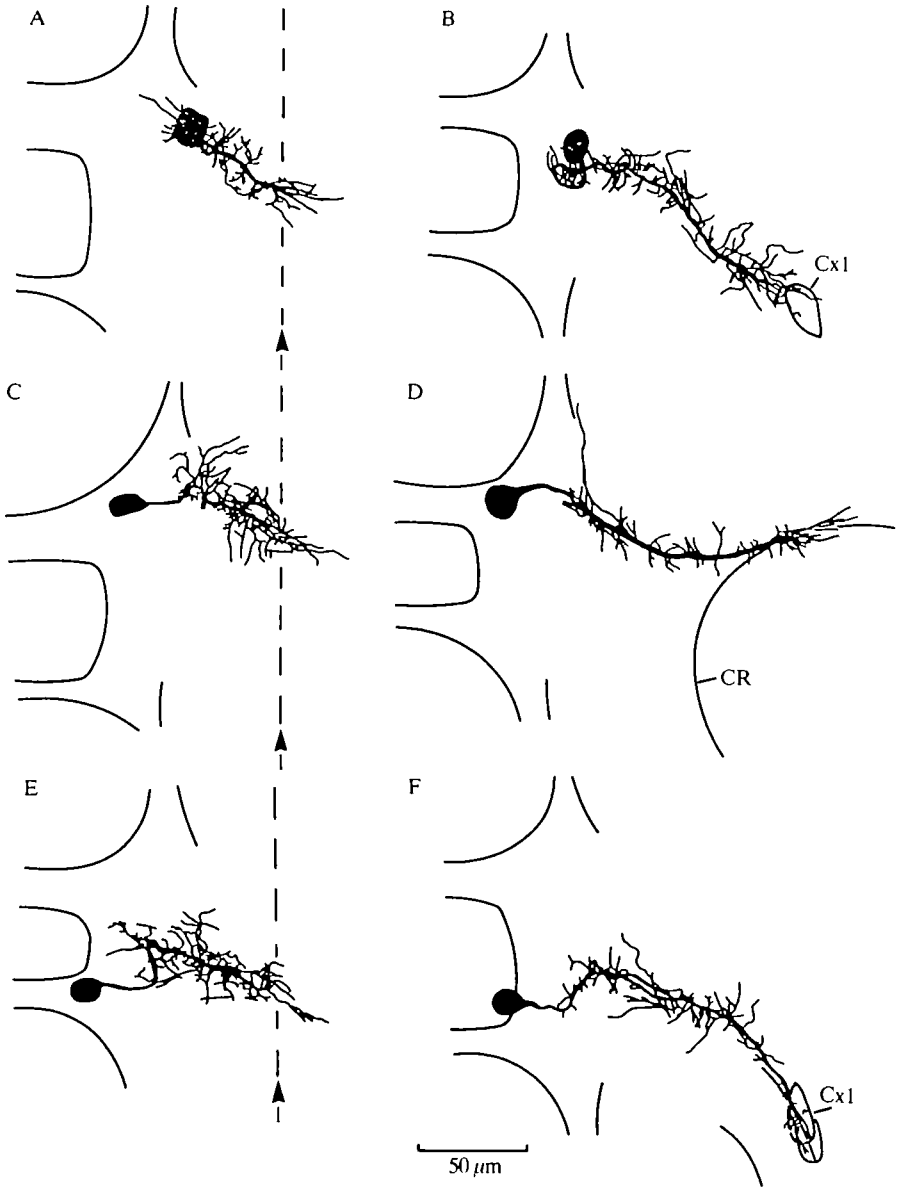

Fig. 4. (A) Motor neurone 3 at 33% of development; (B) motor neurone 3 at 35–36% of development; (C) motor neurone 4 at 33–34% of development; (D) motor neurone 4 at 34–35% of development; (E) motor neurone 5 at 34% of development; (F) motor neurone 5 at 36–37% of development. All drawings were made from LY fills with a camera lucida.

Motor axon 4

Motor axon 4 reaches the edge of the CNS at about the same time as motor axon 3 but its axon is the most anterior in the motor axon array (Fig. 4C). By 35% motor axon 4 has begun growing along nerve root 3, fasciculating closely with motor axon 1 (Fig. 4D).

Motor axon 5

Motor axon 5 is the most posterior in the motor axon array as it approaches the edge of the CNS (at 35% of development, Fig. 4E). By 36–37% the growth cone has arrived at the Cx1 somata while the axon is closely associated with the earlier nerve 5 axons (Fig. 4F).

The position of the motor axon 5 soma enables it to be identified unambiguously as the anterior inhibitor, CI_2 (Watson *et al.* 1985, Whitington & Seifert, 1981).

Later motor axons

Other motor axons follow behind these first 5 axons in quick succession. These axons grow dorsal to the first axons, thereby establishing a second layer of axons beneath the dorsal basal lamina. At the edge of the CNS these axons diverge into either the nerve 3 or nerve 5 pathways. While these later axons have not been individually identified, they include some neurones, which, from the posteriolateral location of their somata, can be recognized as nerve 4 neurones (data not shown). These axons lie posteriorly in the array on the basal lamina. At least at early stages of development these nerve 4 motor axons leave the CNS by the same route as the nerve 5 motor axons.

Discussion

The axons that pioneer a nerve pathway play a pivotal role in axon guidance because of the tendency of late axons to follow the pioneers (Bate, 1978). This study has identified the earliest motor neurones to send axons

out of the segmental nerves in thoracic ganglia of the locust embryo. Two of these neurones (motor axons 1 and 5) can be recognized as known adult motor neurones (neurones SETi and CI_2, respectively).

The patterns of growth displayed by these pioneering motor axons allow us to infer the nature and degree of specificity of the cues that guide these axons. The current study suggests that a small number of cues of broad specificity (i.e. to which large groups of motor axons respond) may be sufficient to guide the motor axons of the segmental nerves up to and out of the edge of the CNS. This view of axon guidance is similar to one that has emerged from recent observations of sensory axon growth in the grasshopper leg (see Caudy & Bentley, 1986*a*,*b*).

The early growth of the pioneering axons can be divided into three phases: growth from the soma to the dorsal basal lamina; posteriolateral growth along the basal lamina in a constant direction; and divergence at the edge of the CNS into either of two pathways, the future nerve root 3 or root 5. Different guidance mechanisms appear to operate in these three phases.

The position of the growth cone with respect to the motor neurone soma when it first contacts the basal lamina suggests that all motor axons initially grow straight upwards in a dorsal direction as do the axons of interneurones (Bastiani *et al.* 1986). This initial polarity may be intrinsic to the motor neurone, determined by the plane of division of the neurone precursors, the ganglion mother cell and the neuroblast. Such a mechanism has been suggested for peripheral pioneer neurones in the grasshopper embryo (Lefcort & Bentley, 1987).

Having contacted the basal lamina, all of the pioneering motor axons show a common pattern of growth: they advance posteriolaterally along the basal lamina in a parallel array. As a result, each motor axon comes to occupy a particular position in that array. An economical explanation of this behaviour is that motor axons are guided by an anteromedial to posteriolateral gradient in the affinity of the basal lamina for motor neurone growth cones. A mechanism of this type has been advanced to explain the initial growth of peripheral pioneer axons towards the CNS (Caudy & Bentley, 1986*a*). In this region the axons grow parallel to the proximal–distal axis of the limb bud, apparently guided by a proximal increase in the affinity of the limb epithelium for pioneer growth cones.

Whatever cue guides the motor axons laterally along the basal lamina, it is specific to motor neurones i.e. interneurones do not respond to it. On the other hand, it is apparently recognized equally well by all motor neurones which send axons out the segmental nerves. There is therefore no need to suppose that the basal lamina possesses specific guidance cues for each individual type of motor neurone.

At the edge of the CNS the earliest motor axon diverges from its straight-ahead growth along the basal lamina and turns sharply anteriorly, an event preceded by the alignment of long filopodia along the same pathway. The path followed by the growth cone of this neurone appears to coincide with the border between the body wall and the base of the coxa and it may therefore be oriented by this segmental boundary. This behaviour recalls that shown by peripheral pioneer axons when they encounter a segmental boundary within the limb bud (Caudy & Bentley, 1986*a*). At this point the axons grow circumferentially around the boundary and are only able to cross it after their filopodia contact the somata of other peripheral pioneers on the other side of the boundary. Caudy & Bentley (1986*a*) suggest that pioneer axons behave in this way because they encounter a discontinuity in a gradient of adhesivity or more generally of growth cone 'affinity' for the limb bud epithelium. Whatever cue motor axon 1 is responding to, it too is apparently closely associated with the epithelium, as the axon is in close contact with the epithelium as it turns anteriorly.

Motor axon 2 behaves quite differently to motor axon 1 at the edge of the CNS: it does not turn anteriorly but continues along its earlier course, which leads it into the coxa. Motor axon 2 may be guided into the coxa by the filopodia or axons of the peripheral pioneer neurones, with which it closely associates. Alternatively it may be following a cue on the epithelium to which the peripheral pioneer axons also orient. The fact that in some cases motor axon 2 meets the peripheral pioneer axon after it has begun to grow along the nerve 5 route argues for the latter possibility.

The observations that motor axon 2 follows closely behind motor axon 1, and that its filopodia explore the same terrain as the latter, suggest that the differential choice by these two axons at the edge of the ganglion results from the active recognition by these two axons of different guidance cues. One would therefore predict that motor axons 1 and 2 should bear different molecular markers, those on the former specific to epithelium, those on the latter to peripheral pioneer axons or a specific marker on the epithelium coincident with these axons.

Motor axons 1 and 2 make this choice reliably: motor axon 1 was never observed to grow down nerve root 5 and motor axon 2 was never found in nerve root 3. This contrasts with the frequent errors in pathway choice which at least one of these motor axons, SETi, makes further in the periphery (Myers *et al.* in preparation).

On reaching the edge of the CNS the other early motor axons follow either motor axon 1 or motor axon 2, associating closely with these pioneering axons. Like the pioneers the follower motor axons make this choice reliably: a given axon is always found in the same pathway.

In summary a limited set of molecular markers may be sufficient to guide the growth of all segmental motor axons from their somata up to the edge of and out of the CNS. Afferent axons may possess the same markers and respond to the same guidance cues as motor axons, further economizing on the amount of genetic information needed to generate the mature pattern of axonal projections.

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