Development 118, 449-461 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993



# Evolutionary change in neural development within the arthropods: axonogenesis in the embryos of two crustaceans

**Paul M. Whitington[1], David Leach[1] and Renate Sandeman[2]**

[1]Department of Zoology, University of New England, Armidale, NSW, 2351, Australia
[2]School of Biological Sciences, University of New South Wales, Kensington, NSW, 2033, Australia

## SUMMARY

**It has been previously suggested that there is a conservative program for neural development amongst the arthropods, on the basis that a stereotyped set of cells involved in establishing the axon tracts in the CNS of insect embryos is also present in crayfish embryos. We have examined the spatiotemporal pattern of axon growth from a set of early differentiating central neurons in the embryo of two crustaceans, the woodlouse *Porcellio scaber* and the freshwater crayfish *Cherax destructor*, and drawn comparisons with insect neurons whose somata lie in corresponding positions within the CNS. While many of the woodlouse and crayfish neurons show a similar pattern of axon growth to their insect counterparts, the axon trajectories taken by others differ from those seen in insects. We conclude that this aspect of early neural development has not been rigidly conserved during the evolution of the crustaceans and insects. However, the extent of similarity between the insects and the crustaceans is consistent with the idea that these groups of arthropods share a common evolutionary 'Bauplan' for the construction of their nervous systems. While the pattern of early axon growth in the woodlouse and crayfish embryos is sufficiently similar that many neurons could be confidently recognised as homologues, several differences were noted in both the relative order of axon outgrowth and axon morphologies of individual neurons.**

Key words: crustacean embryo, CNS development, Isopoda, woodlouse, crayfish, Decapoda, axon growth, arthropod evolution, neuron morphology, insect embryo

## INTRODUCTION

The relationship between development and evolution has attracted the attention of biologists for over a century (Gould, 1977). Early workers in this field were struck by the observation that early embryonic events tend to be conserved during evolution and that differences between related organisms generally arise as a result of modifications to relatively late developmental processes (reviewed by Raff and Kaufman, 1983; Gould, 1977). On the other hand, there are a number of well documented cases of radical change during early development in closely related organisms (e.g. Dohle and Scholtz, 1988; Harvey and Raff, 1990; Wray and Raff, 1990). Such observations raise a number of questions. Why are early developmental processes conserved in some cases while in others they are open to change? Are there any evolutionary trends in the developmental changes that do occur and to what extent do these trends stem from intrinsic properties of developmental programs?

A striking example of evolutionary conservatism is provided by the early development of insect central nervous systems (CNS). A set of early differentiating neurons can be recognized in the CNS of the grasshopper embryo, which is also present, virtually unchanged, in the CNS of the evolutionarily distant fruitfly and moth embryos (Goodman et al., 1984; Thomas et al., 1984). These neurons can be confidently considered homologous, as at least 9 neurons, which occupy corresponding positions in the different insects, have identical axon morphologies and also show very similar temporal sequences of axon outgrowth, (although some minor differences exist in the latter respect, (Jacobs and Goodman, 1989). It therefore appears highly likely that there is a conserved basic plan within the insects for the development of CNS axon pathways.

Thomas et al. 1984 additionally reported that this set of central neurons, which is conserved in the insects, is also present in the embryo of the freshwater crayfish *Procambarus* and therefore concluded that 'all arthropod nervous systems seem to be constructed using the same embryonic plan'. It seems very unlikely that a set of 9 neurons in corresponding positions in the insect and crayfish nervous system could have convergently acquired identical morphologies. The claim that these insect and crustacean neurons are homologous therefore appears to be well-founded. More recently this finding was advanced, together with a reported strong similarity between insect and crayfish embryos in the pattern of neural expression of the segmentation gene *engrailed* (Patel et al., 1989), as evidence that these two groups 'share a relatively recent common

ancestor', showing that this finding has considerable phylogenetic, as well as developmental significance.

We have recently found that the embryo of the centipede *Ethmostigmus rubripes*, a representative of the myriapods, the arthropod group widely held to be most closely related to the insects, shows little resemblance to insects in its mode of formation of CNS axon tracts (Whitington et al., 1991). In this arthropod, the pioneering axons in the CNS arise from neurons located in the brain, with neurons located in the segmental ganglia beginning axonogenesis only later in embryogenesis. Furthermore, the pattern of axon growth from segmental neurons in the centipede has little in common with that reported in insects.

Given these recent findings in the myriapods and the importance of the conclusions reached by Thomas et al. (1984), we considered it necessary to readdress the question of conservatism between the insects and the crustacea in nervous system development. Specifically, we sought to determine whether the set of neurons that is strongly conserved during insect evolution, is present unchanged in crustaceans. To this end, we have examined early neural development in the embryos of two crustaceans, the woodlouse *Porcellio scaber*, an isopod, and the Australian freshwater crayfish *Cherax destructor*, a decapod.

## MATERIALS AND METHODS

Gravid woodlice *Porcellio scaber* (Latrielle) were collected during their breeding season from September to March at Armidale and Guyra, NSW, Australia. Outside of the natural breeding season, breeding colonies of 20-100 animals were maintained in plastic boxes at 20°C with 16 hour light and 8 hour darkness. Females incubate 20-60 embryos in a marsupium on the ventral side of the thorax. The oostegites of the marsupium were raised with a blunt dissecting needle and several embryos were removed without damaging the female. Hence successive stages of development could be observed from the same adult. Embryos that had been removed from the marsupium were maintained in a healthy condition in crustacean saline (12 g/l NaCl, 0.4g/l KCl, 0.25 g/l $MgCl_2.6H_2O$, 0.2 g/l $NaHCO_3$, 1.5 g/l $CaCl_2.2H_2O$) for several days at 4°C.

Embryonic development of *Porcellio scaber*, from the first appearance of the egg in the marsupium to hatching from the vitelline membrane, requires approximately 26 days at 20°C (unpublished observations). The hatched young remain in the marsupium for a period of several days before emerging. We examined embryos from several developmental stages ranging from 45% to 90% of embryonic development. A short description of some of the major transformations in external morphology during this period follows.

The 45% stage is marked by the apppearance of limb buds, visible under the dissecting microscope as small bumps on the ventral side of the embryo.

At the 50% stage the limb buds and yolk sacs are clearly visible and the yolk begins to become enclosed in a pair of yolk sacs.

At 60% the hindgut has begun to extend anteriorly. Approximately 3/4 of the yolk is enclosed within the yolk sacs. The embryo hatches from the chorion.

By 70% of embryonic development, yolk enclosure is complete and the dorsal body wall has formed. Movement of the embryo is apparent.

By 80%, the embryo has rotated in the dorsoventral axis within the vitelline membrane and pigment appears on the margins of the tergites.

At the 90% stage the eyes show a mottled red pigmentation.

Hatching takes place at 100% but the young larvae remain within the marsupium for some time.

Embryos of the Australian freshwater crayfish *Cherax destructor* were obtained from berried females maintained in laboratory cultures. Breeding pairs were kept in tanks of de-chlorinated water at 19°C with 16 hours light and 8 hours darkness. The embryos are carried on the abdomen of the female attached to the swimmerets and are easily removed without damage. Embryos continue to develop normally in isolation. Embryonic development takes 40 days at 19°C (Sandeman and Sandeman, 1991).

For neuron injections, embryos were dissected from the chorion and vitelline membranes and the limb buds were removed. The embryo was placed in saline, ventral side down, on a poly-L-lysine coated microscope slide, to which it adhered strongly. The yolk sacs and/or gut tube were removed and the body wall flattened out to expose the central nervous system.

Intracellular injections were done under Nomarski optics using microelectrodes pulled on a David Kopf or Brown-Flaming (Sutter Instrument Co.) microelectrode puller. Electrodes were backfilled with a 5% solution of Lucifer Yellow (LY) in double distilled water. The LY was injected with a 0.2 nA DC hyperpolarizing current applied for 2 minutes.

LY-filled embryos were fixed for 45 minutes in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). After washing in PBS for 1 hour, embryos were incubated overnight at 4°C in polyclonal rabbit anti-LY antiserum diluted 1:250 in PBT (PBS, 0.25% bovine serum albumin, 0.4% Triton X-100). Following a 1 hour wash in PBT, embryos were incubated for 3 hours at room temperature in HRP-conjugated donkey anti-rabbit Ig (Amersham) diluted 1:500 in PBT. Staining was obtained by a standard DAB reaction and after a 1 hour wash in PBS, embryos were cleared and mounted in glycerol.

DiI stains of nerves were obtained by backfilling electrodes with a 0.4% solution of DiI in ethanol and depositing a small crystal of DiI in the intersegmental nerve by passing a 2 nA DC depolarizing current for 1-5 minutes. Preparations were left for up to 3 hours to allow the dye to reach the somata. DiI fluorescence was visualized using incident light with a 546 nm exciter filter and a 610 nm barrier filter. Images were captured using a video camera (Pulnix, USA) and a Neotech frame grabber in an Apple Macintosh IIci computer.

Rhodamine phalloidin staining was carried out on fixed, dissected embryos by incubation in the dark, for 1 hour in rhodamine phalloidin diluted in PBT (Molecular Probes, 2 Units in 200 µl PBT). Embryos were washed in PBS, mounted in 75% glycerol, 25% PBS and examined using DiI fluoresence filters. Images were recorded in the same way as DiI stained preparations.

## RESULTS

### Gross morphology of the CNS of woodlouse and crayfish embryos

The mature embryonic ventral nerve cord in both the woodlouse and the crayfish consists of a pair of longitudinal connectives, with a single anterior and posterior commissure and paired segmental and intersegmental nerves in the ganglion of each segment, a pattern which is similar to that seen in the winged insects. The pattern of axon tracts is clearly displayed in embryos stained with rhodamine-phalloidin (Fig. 1A,B).

The woodlouse ventral nerve cord consists of three subesophageal ganglia, seven thoracic and seven abdominal ganglia. The first thoracic ganglion innervates the maxillipeds.

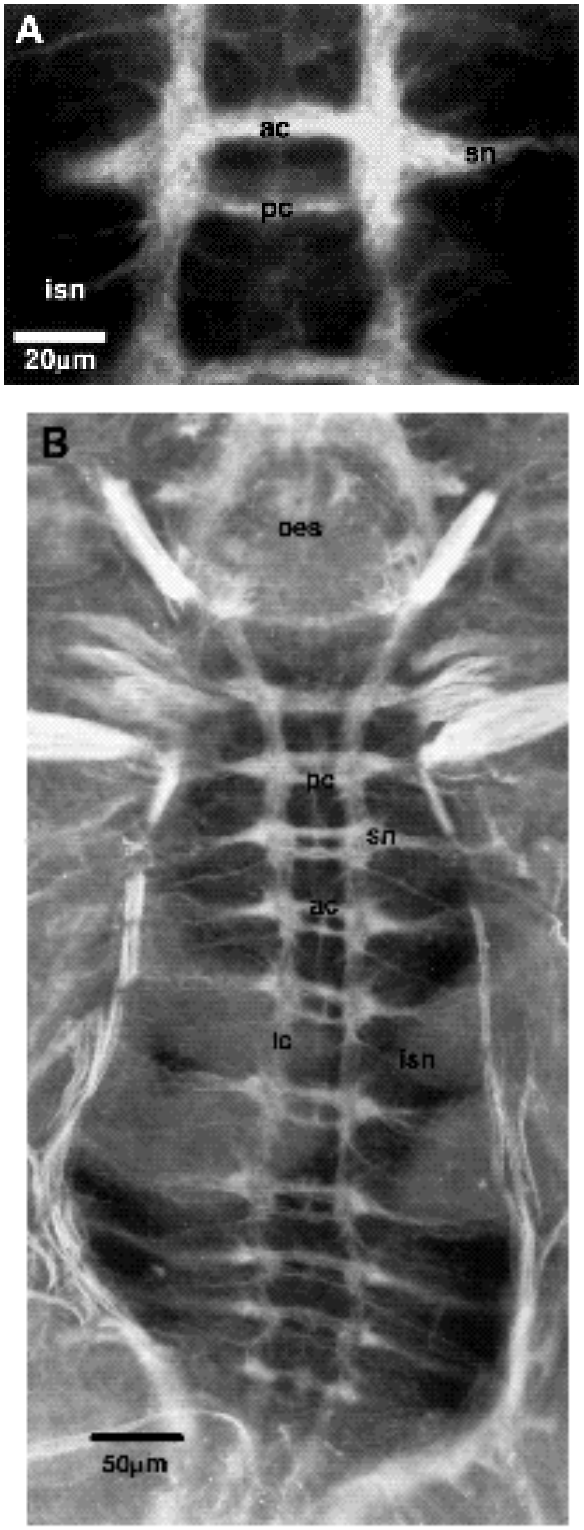



**Fig. 1.** Photomicrographs of the pattern of central axon pathways in the woodlouse (A) and crayfish (B) embryos at 55% of development, revealed by staining with rhodamine-phalloidin. A single thoracic segment is shown in the woodlouse preparation while the chain of suboesophageal (oesophagus, oes) and thoracic ganglia is evident in the crayfish embryo.The longitudinal connectives (lc), anterior commissure (ac), posterior commissure (pc), segmental nerve (sn) and intersegmental nerve (isn) are evident.

The remaining thoracic ganglia supply the six pairs of legs. An additional segment appears late in embryonic development, and gives rise post-embryonically to a seventh pair of walking legs. The crayfish embryo possesses 3 suboesophageal ganglia, 8 thoracic and 6 abdominal ganglia. Observations were restricted to the first to sixth thoracic ganglia for the woodlouse embryo and to the first to 5th thoracic ganglia for the crayfish. No significant variation in axon morphology between these segments was observed for the neurons examined in this study.

### Arrangement of somata in the CNS

Somata in the CNS can be clearly visualized in both the woodlouse and crayfish embryo under Nomarski optics. The arrangement of these somata is relatively constant between embryos of the same age, and up to late stages of embryogenesis, individual somata can be reliably identified from soma position alone. In this study, we concentrated our attention on somata in the most dorsal layers of the CNS between the region bounded laterally by the longitudinal connectives. Most of the pioneering axons in the CNS appear to arise from neurons in this region and it has been intensively explored in insect embryos. Fig. 2A,B shows the arrangement of these somata in the dorsal-most layers of the CNS at 50% of development in the woodlouse and crayfish embryos, respectively. The preparation from which Fig. 2A was drawn is shown in Fig. 5. We have used similarities in soma position, with respect to surrounding cells, and in axon morphology (see below) to assign a common label to a given neuron in the two species. The common lettering of a neuron in the crayfish and the woodlouse does not necessarily imply homology: the issue of homology of neuron identity between the species is addressed in the Discussion.

As development proceeds and new cells and neurites are added to the CNS, some dorsal neurons are pushed into more ventral layers of the ganglion, or are displaced in the anteroposterior or medial-lateral axes. These rearrangements are more pronounced in the crayfish embryo than in the woodlouse embryo, resulting in major differences between these species in somata arrangement at late embryonic stages.

### Pattern of axon outgrowth from central neurons

To establish when axons first arise in the CNS, we used rhodamine-phalloidin staining of whole embryos. In the woodlouse, the first central process revealed by this method appears well in advance of all other central axons, at 45% of development. Lucifer Yellow injections confirm that the process originates from a large cell in the midline of the mandibular segment, which has additional, anteriorly directed cellular extensions in a Y-shaped arrangement (Fig. 3). The posterior process grows along the dorsal side of the CNS, arborizing extensively in more posterior segments. The ultimate fate of this process is uncertain but it may be the founder of a median nerve which is evident later in embryogenesis. We have seen a similar central process in the crayfish embryo, but have not as yet identified the neuron from which it originates.

In the crayfish embryo the first sign of axons in the longitudinal connectives is at around 40-45% of development.

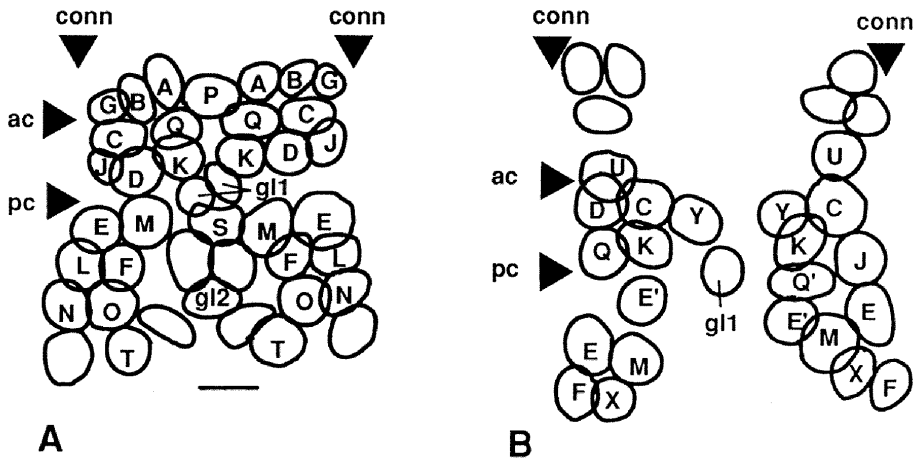



**Fig. 2.** Camera lucida drawing of dorsal somata in 50% embryos of the woodlouse (A) and crayfish (B). Axon morphology has been established for the labelled cells. Scale bar, 10 μm. Arrow heads show the future positions of the connectives and the commissures. The embryo from which Fig. 2A was prepared in shown in Fig. 5.

Intracellular injections of these first axons indicate that they originate, not from neurons in segmental ganglia, but from neurons located in the brain (Fig. 4). These axons enter segmental ganglia at around the time that neurons in those ganglia are beginning axonogenesis.

Phalloidin staining of woodlouse embryos reveals the first central axons in other segments at around 50% of development. These first appear in the most anterior segments and arise from neurons in the most dorsal layers of the CNS. Intracellular fills of more ventrally located neurons show that these begin axonogenesis later than dorsal neurons. There is a clear anteroposterior sequence of axon growth. The figures for percentage of embryogenesis given in the descriptions below apply to the most anterior thoracic segments. The same events take place in more posterior segments later in development.

A similar mode of axon growth is seen in the crayfish embryo, except that the axons from segmental neurons grow out after and fasciculate with the pioneering longitudinal axons from the brain. The first axons from central neurons

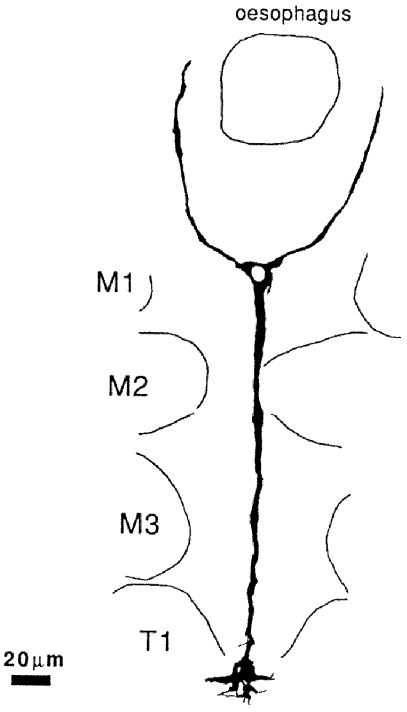



**Fig. 3.** Camera lucida drawings of a LY-injected neuron in the mandibular segment (M1) of a 45% woodlouse embryo. This cells sends a median process posteriorly in advance of the appearance of other axons in the CNS. M2, M3, T1 indicate the bases of the limb buds for the mandibular, first maxillary and second maxillary segments, and first thoracic segments, respectively.


**Fig. 4.** Photomicrograph of an early outgrowing brain neuron in a 40% crayfish embryo, which has been injected intracellularly with LY. Its soma (s) is located ventrally in the brain, just anterior to the oesophagus (o). The growth cone (g) has reached the mandibular segment. Scale bar, 50 μm.

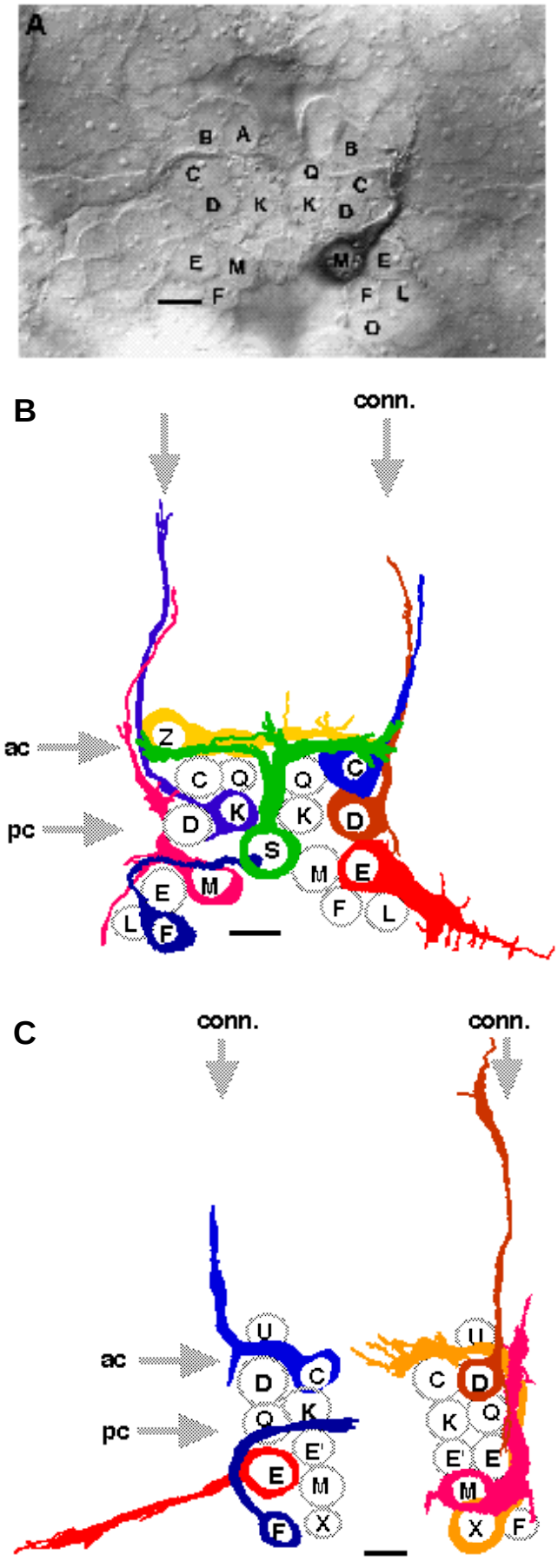



**Fig. 5.** (A) Photomicrograph of LY-injected neuron M in a 50% woodlouse embryo. The axon of this neuron runs in between the somata of neurons D and E. Long filopodia extend anteriorly from the growth cone. This micrograph also shows the regular arrangement of somata, drawn in Fig. 2A. Nomarski optics. Scale bar, 10 μm. (B,C) Camera lucida drawings of LY-filled neurons involved in pioneering of central axon pathways in the woodlouse (B) and crayfish (C). These drawings were made from different embryos between 50 and 55% of development and superimposed to show the spatial relationships between these axons and surrounding somata. Scale bar, 10 μm.

in anterior segments of the crayfish embryo appear at about 45% of development. Differences between segments in the extent of axon growth are even more marked in the crayfish embryo than in the woodlouse embryo.

## Segmental axon growth in woodlouse embryo

LY fills of pairs of neurons on opposite sides of a given segment indicate that the first cells to contribute axons to the central scaffold in the woodlouse embryo are E,M,S and Z; these cells all appear to begin axonogenesis at about the same time.

Neuron M (number of neurons filled, *n*=20) pioneers the longitudinal connectives. A single, broad, lammelipodial process consistently extends from the anterolateral region of the soma and advances between the region of contact of the neighbouring neurons D and E. This process, or filopodia arising from it, enwrap the lateral and medial faces of cell E and it subsequently thins into an axon, which grows anteriorly, eventually extending to the brain. Typically, a small group of long (up to 50 μm) filopodia extend anteri-

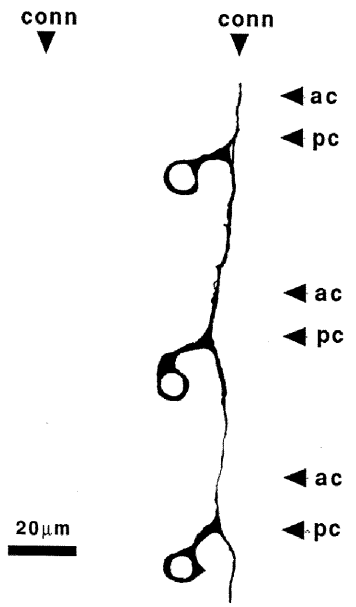



**Fig. 6.** Camera lucida drawing of LY-filled neuron M in three successive segments of a 55% embryo. The anteriorly directed axon of each M neuron fasciculates with the posteriorly directed process of its homologue in the next segment. The positions of the anterior and posterior commissures and the connectives are indicated by arrowheads.

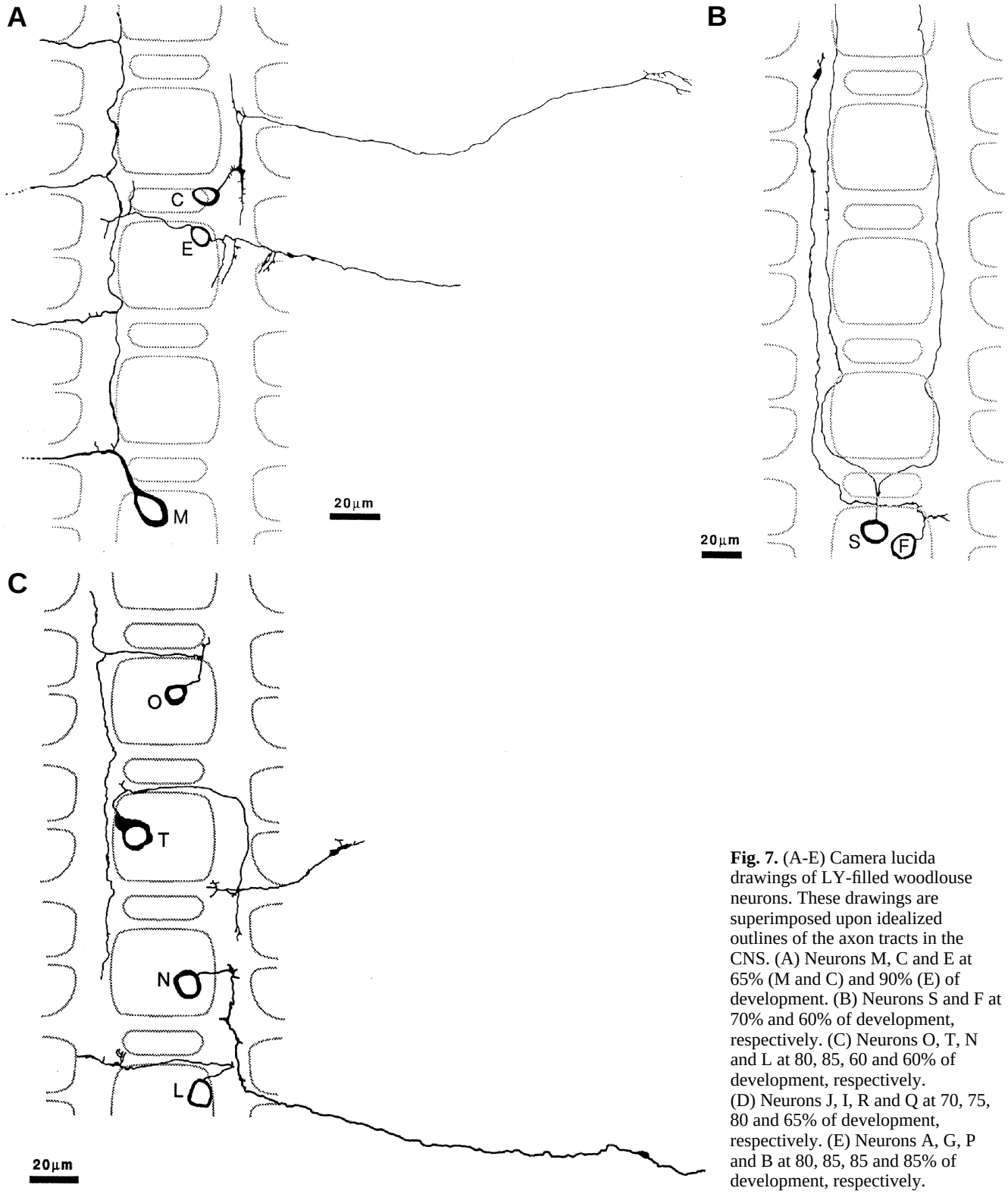
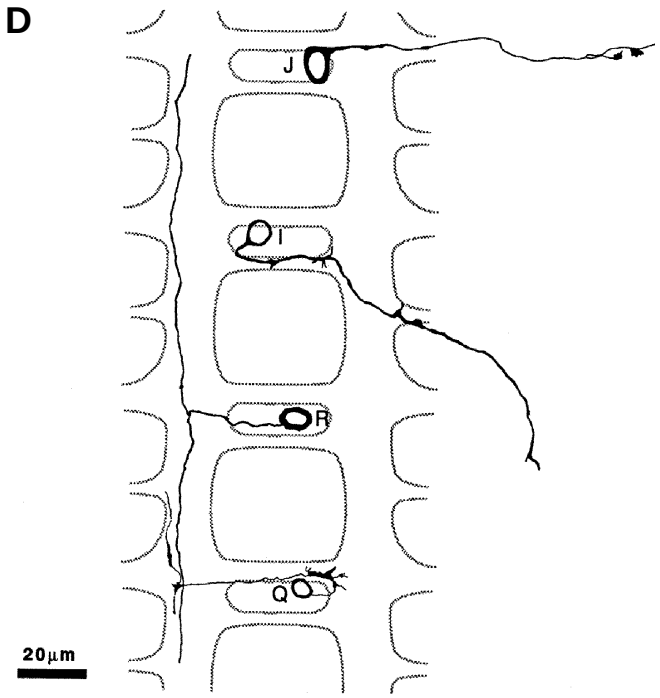
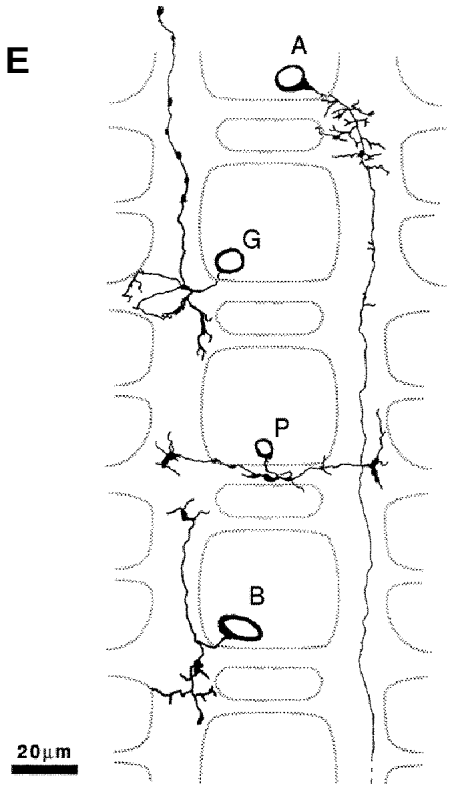



**Fig. 7.** (A-E) Camera lucida drawings of LY-filled woodlouse neurons. These drawings are superimposed upon idealized outlines of the axon tracts in the CNS. (A) Neurons M, C and E at 65% (M and C) and 90% (E) of development. (B) Neurons S and F at 70% and 60% of development, respectively. (C) Neurons O, T, N and L at 80, 85, 60 and 60% of development, respectively. (D) Neurons J, I, R and Q at 70, 75, 80 and 65% of development, respectively. (E) Neurons A, G, P and B at 80, 85, 85 and 85% of development, respectively.

orly from the tip of the growing axon (Fig. 5A,B). (The relative lengths of axons in Fig. 5B do not represent the order of axon outgrowth from these cells, as the figure is constructed by superimposing neurons at different stages over the range 50-55%). The posteriorly directed process on the lateral side of cell E is eventually retracted but persists for at least the next 10% of development and in some cases grows some distance further posteriorly. The advancing anterior axon of the M neuron in the next most posterior segment contacts this posterior process and, in this




way, the connection between adjacent ganglia is established (Fig. 6). Posteriorly directed axons from other neurons do not develop until after this connection has been made. Later in development (from around 60% onwards), the M neuron extends collateral processes out of the segmental nerve in every segment through which the main axon courses (Fig. 7A).

Cell E (*n*=13) pioneers the intersegmental nerve. It extends a broad lamellipodium with short (5-10 μm) filopodia in a posterolateral direction (Fig. 5B). The lamellipodium subsequently thins into an axon which continues to extend into the periphery along its original course. Later in development, around 55%, a medially directed process extends from neuron E, crosses the midline in the posterior commissure and arborizes in the contralateral neuropile in the vicinity of the contralateral E neuron. By 90% of development, dendritic branches have begun to extend from the neurite in the ipsilateral region of the neuropile (Fig. 7A).

Cell Z (*n*=3) produces a medially directed process, which pioneers the lateral portion of the anterior commissure (Fig. 5B). As the lamellipodium thins to an axon it meets the laterally growing axon from cell S (see below), thereby completing the anterior commissural pathway. It then advances across the midline, fasciculating with the axon of its contralateral homologue. The trajectory taken by the Z axon corresponds to the line of contact between the cell row A, B, G with the cells C and Q.

Cell S (*n*=9) is one of a group of unpaired median neurons. It sends a single axon anteriorly along the dorsal midline. At the site of the future anterior commissure, the axon bifurcates (Fig. 5B) and each branch grows laterally, meeting the medially directed axon of cell Z on each side. When the S axons meet the longitudinal connective pathway (previously pioneered by M), they grow anteriorly, extending to the brain by 70% of development (Fig. 7B).

Axons M, E, Z and S are followed closely by those of neurons C (*n*=10), D (*n*=10) and K (*n*=6). Each of these axons grows from the anterolateral side of the soma and advances anteriorly. Axons M, D and K run along the medial border of the connective and fasciculate with each other. Axon K grows in between neurons C and D before turning anteriorly while axon D initially extends along the lateral face of cell C (Fig. 5B). Both D and K continue to advance anteriorly along the connective, D extending as far as the brain by 75% of development.

Axon C crosses from the medial to the lateral side of the connective as it advances anteriorly (Fig. 5B). In some cases, C also forms a posteriorly directed branch, which follows the posterior branch of axon M, and an additional posterior branch further along the axon (Fig. 7A). When axon C reaches axon E in the next most anterior segment, it follows this axon out of the intersegmental nerve. In some cases, it also sends a short medial branch back towards neuron E or further anteriorly along the connective. The additional branches are found more frequently at later stages of embryogenesis, at which time they display fine subbranches (Fig. 7A), which may develop into the dendritic arborizations of this neuron.

Neuron F (*n*=10), the pioneer of the posterior commissure, begins axonogenesis shortly after C and D. Its axon grows anteriorly, around the lateral side of neuron E (Fig. 5B), before turning medially to found the posterior commissure between 50 and 55% of development. Thus, the posterior commissure begins to form after the anterior commissure. Axon F turns anteriorly when it encounters the contralateral longitudinal connective (Fig. 7B) and extends to the brain by 80% of development.

Axon F is closely followed by the axon of cell O (*n*=9), which begins axonogenesis around 55% of development.

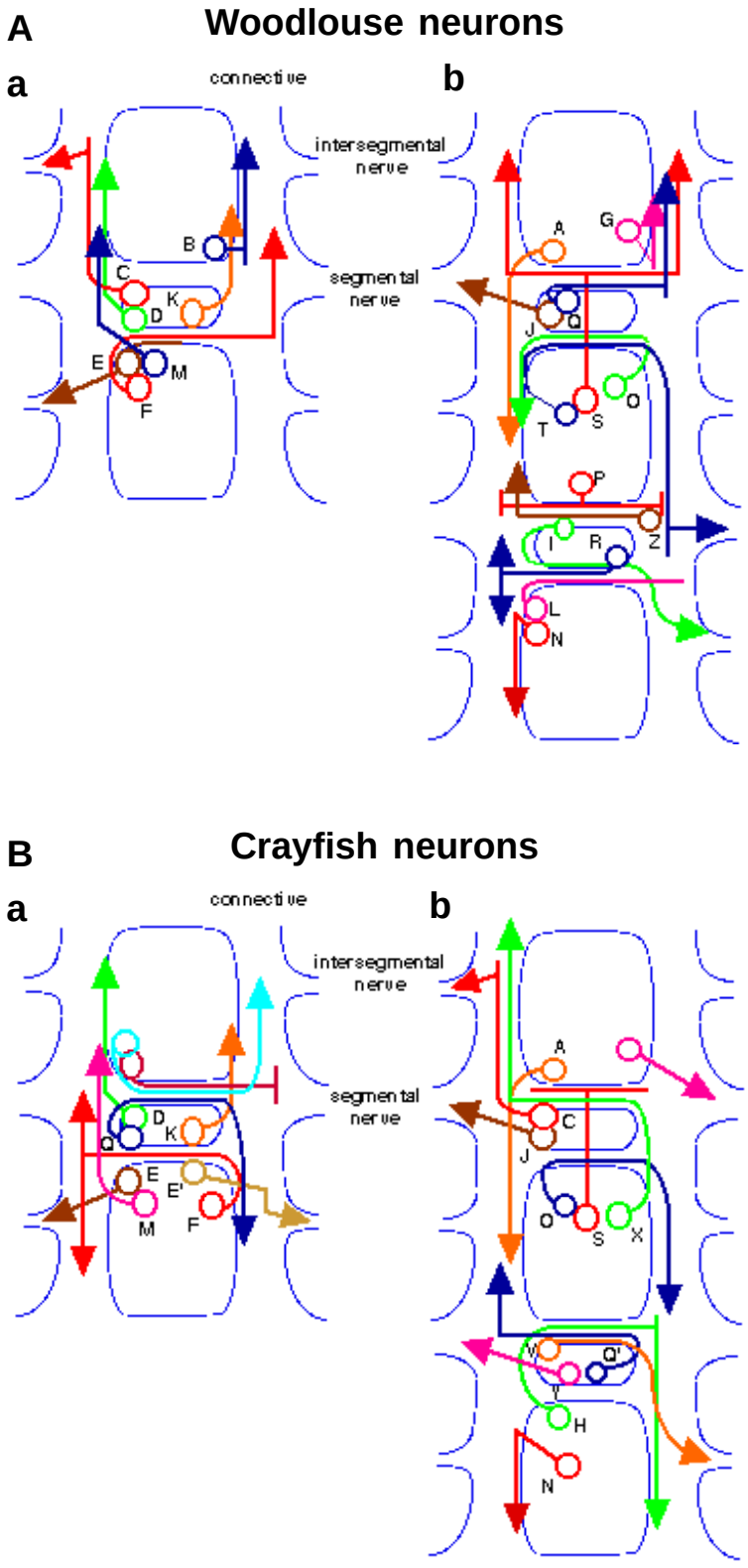



**Fig. 8.** Schematic diagram of axon morphologies of central neurons examined in this study in the woodlouse (A) and crayfish (B) embryo. To minimize overlap, a different subset of the segmentally iterated neurons studied is shown in each of three segments for both species. One segment is drawn in part a of both A and B, while two neighbouring segments are represented in part b of the same figure. The cells shown in (a) lie dorsal to those in (b). Arrowheads indicate that the axon continues along the course indicated.

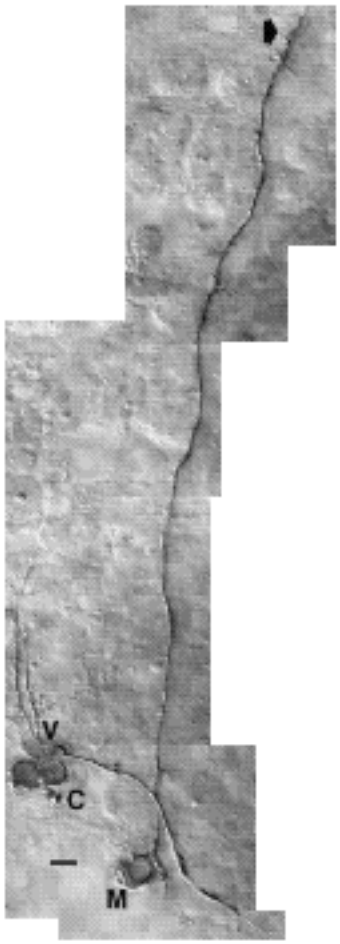



**Fig. 9.** Photomontage of a 60% crayfish embryo in which neurons V, C and M have been intracellularly injected with LY. The growth cone of neuron M is indicated by an arrowhead. Nomarski optics. Scale bar, 10 μm.

This axon initially grows anteriorly then turns into the posterior commissure, posterior to axon F. A short branch is extended anteriorly from the site of this turn. On reaching the contralateral longitudinal connective, axon O turns and extends posteriorly. In some embryos a short anteriorly directed branch is also formed at this turn (Fig. 7C).

Cell L ($n$=4), which lies just lateral to cell F, also sends an axon into the posterior commissure, at about the same time as axon O. However, axon L initially takes an anterolateral trajectory from the soma, before turning into the commissure (Fig. 7C). It appears to terminate at the base of the contralateral segmental nerve.

The axon of cell T ($n$=1), which lies just posterior to cell O, initially follows a similar course to the axon of the latter cell, turning into the posterior commissure and growing posteriorly down the contralateral longitudinal connective. However, instead of continuing down the connective, axon T turns into and extends down

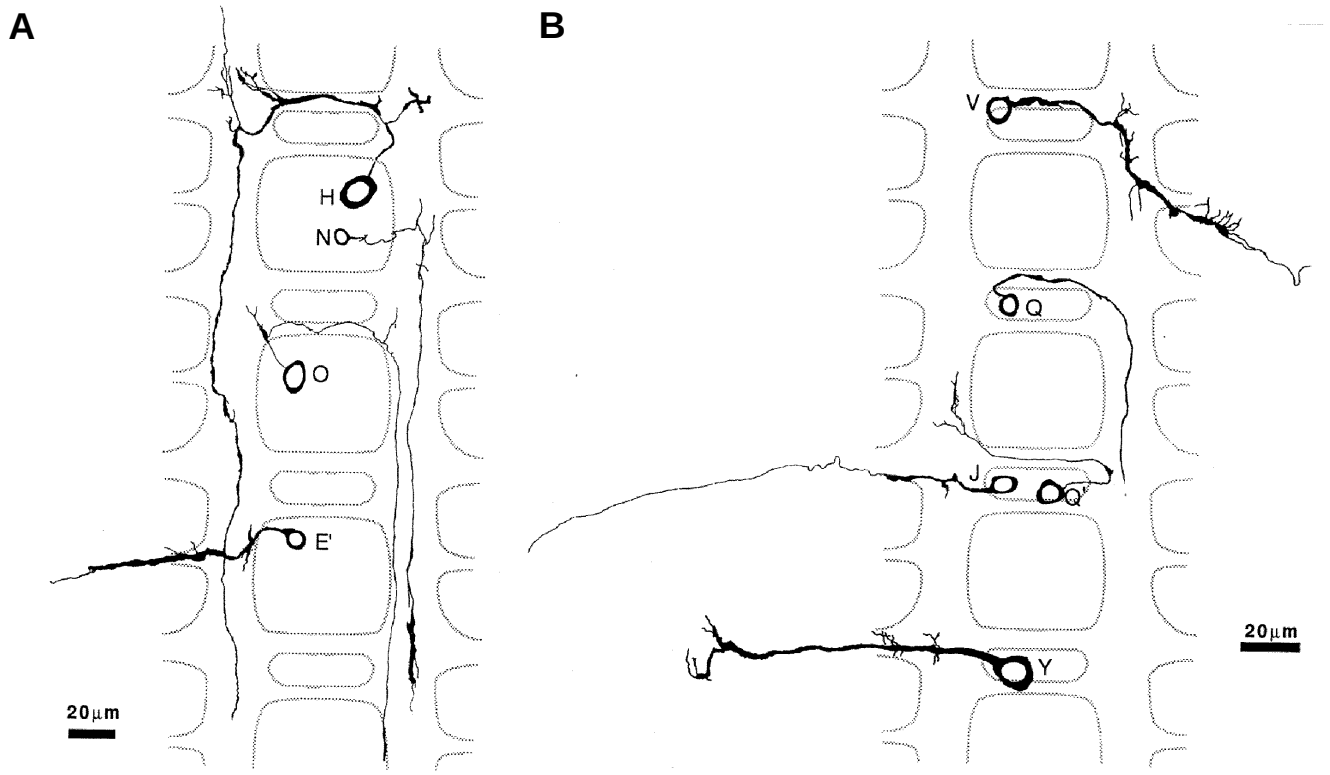



**Fig. 10.** Camera lucida drawings of LY-filled crayfish neurons. These drawings are superimposed upon idealized outlines of the axon tracts in the CNS. (A) Neurons H, N, O and E′ at 70, 65, 70 and 55% of development, respectively. (B) Neurons J, Q, Q′, V and Y at 65, 60, 60, 55 and 65% of development, respectively.

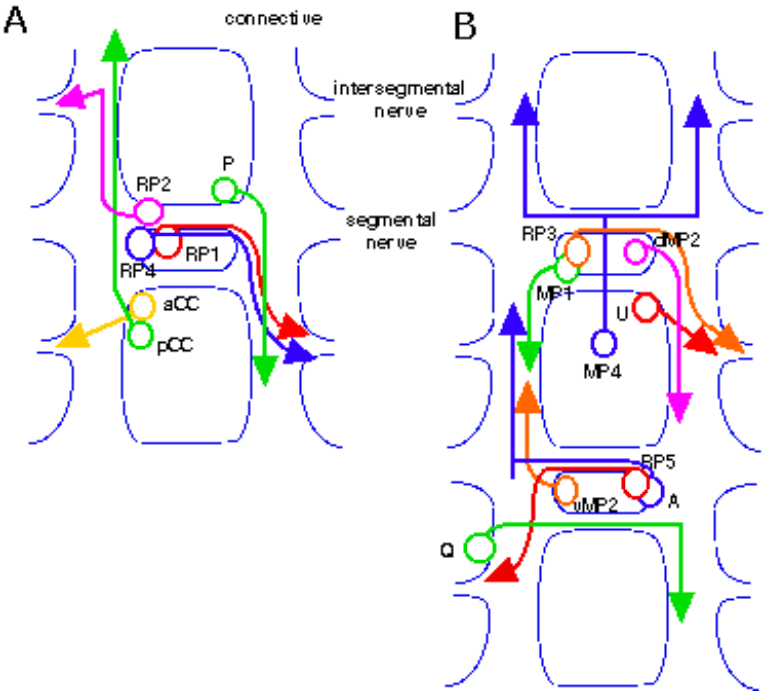



**Fig. 11.** Schematic diagram of axon morphologies of central neurons in insect embryos. Data are drawn from previous studies (see text). One segment is drawn in A, while two neighbouring segments are represented in B. The cells shown in A lie dorsal to those in B. Arrowheads indicate that the axon continues along the course indicated.

the segmental nerve of the next most posterior segment (Fig. 7C). Short, medially and posteriorly directed branches are also formed at this turn.

The axon of neuron N (*n*=5), the remaining cell in the dorsal group of neurons that lie posterior to the posterior commissure, follows a different course from any of the latter neurons. It grows laterally, then turns posteriorly down the longitudinal connective, sometimes extending a short branch into the intersegmental nerve. On reaching the intersegmental nerve of the next most posterior segment, axon N turns into this nerve and extends into the periphery (Fig. 7C). A short medially directed branch may also form at this turn.

Cell Q (*n*=2), which lies adjacent to cells K, D and C at early stages (50%), but is rapidly pushed into a more ventral position, begins axonogenesis somewhat later than the neighbouring cells, at around 55%. It produces a laterally directed axon, which turns anteriorly into the ipsilateral longitudinal connective, then does a U-turn into the anterior commissure (Fig. 7D), and turns anteriorly on contacting the contralateral longitudinal connective. A short posteriorly directed branch may form at this turn.

Three cells, I (*n*=7), J (*n*=5) and R (*n*=2), which lie just ventral to the K, D, C, Q group

at 50%, begin axonogenesis after the latter cells. Axon J is an early contributor to, if not the pioneer of, the segmental nerve. The axon runs directly laterally into the periphery from the anterolateral region of the soma (Fig. 7D). Cell I's axon runs laterally, then loops into the posterior commissure, turns posteriorly down the contralateral longitudinal connective and runs out the intersegmental nerve of the next most posterior segment (Fig. 7D). Cell R's axon runs medially in the posterior commissure, crosses the midline and on reaching the contralateral longitudinal connective, bifurcates and sends branches anteriorly and posteriorly along the full length of the nerve cord (Fig. 7D).

The final set of neurons examined in this study is the dorsal group lying immediately anterior to the anterior commissure at stage 50%, cells A, B, G and P. A ($n$=6) is the first of these cells to begin axonogenesis, at around 55%. A's axon runs laterally around the posterior side of cell B and G. On contacting the ipsilateral longitudinal connective, it turns posteriorly and grows down the nerve chord for at least 4 segments (Fig. 7E). Neuron B ($n$=6) extends an axon laterally into the ipsilateral longitudinal connective, where it turns anteriorly and courses through several segments. A short posteriorly directed branch is also found at this turn (Fig. 7E). G's ($n$=4) axon runs posterolaterally into the ipsilateral longitudinal connective, then turns anteriorly and, by 90% has extended 2 segments along the nerve chord. A number of short branches are formed in the region of the anterior turn (Fig. 7E). Neuron P ($n$=3) is an unpaired midline neuron. Its axon runs posteriorly to the anterior commissure where it bifurcates into two laterally running branches, each of which extends along the anterior commissure to the lateral region of the neuropile, at the base of the segmental nerve (Fig. 7E).

The final embryonic morphologies of these woodlouse neurons are shown schematically in Fig. 8A.

Intracellular filling of dorsal neurons in the rectangle bounded by the connectives and commissures failed to reveal any cells with an axon morphology like the insect RP1/3/4/5 neurons, which occupy the corresponding position in the embryonic insect CNS. Knowing that the insect RP neurons send axons out of the intersegmental nerve via the anterior commissure, we backfilled the intersegmental nerve of the woodlouse, to determine whether equivalents to the insect RP neurons exist in more ventral regions of the woodlouse ganglion. None of the central neurons stained in this way sent an axon into the anterior commissure ($n$=4) and on that basis we conclude that neurons with an RP morphology do not exist in the woodlouse.

## Segmental axon growth in the crayfish embryo

As noted above, we have assigned common labels to certain segmental neurons in the crayfish and woodlouse embryos, using a combination of relative soma position and axon morphology as criteria for equivalence. It follows that a crayfish neuron which has the same label as a woodlouse neuron shows a similar (but not necessarily identical) pattern of axon growth. We indicate below where differences in axon growth exist for neurons with the same labels. Some crayfish neurons do not appear to have counterparts in the woodlouse: these are assigned letters that are not used in the woodlouse.

The first segmental neurons to initiate axonogenesis in the crayfish embryo are M, E, C, D and K; all of these axons grow out at about the same time. E ($n$=38) pioneers the intersegmental nerve, growing directly into the periphery in a posterolateral direction (Fig. 5C). Unlike its woodlouse equivalent, E does not form a contralateral process during embryogenesis.

The axon of neuron M grows around the posterior side of neuron E to enter the longitudinal connective (Fig. 5C), rather than the anterior side of this neuron, which is the trajectory followed by its woodlouse counterpart. This difference correlates with the more posterior relative position of neuron M in the crayfish compared to the woodlouse. A further species difference is seen later in development: the crayfish M neuron does not form the efferent collateral branches present in the post 60% woodlouse embryo (Fig. 9).

Whereas in the woodlouse, axon M clearly leads the axons of C, D and K, in the crayfish none of these axons is a consistent leader of the others. There are minor differences between the crayfish and the woodlouse in the initial course taken by axons C, D and K: these appear to result from differences in the relative positions of the somata of these neurons. For example, neuron C in the crayfish lies more medial than its woodlouse counterpart and thus its axon initially grows around the anterior side of cell D, rather than directly into the longitudinal connective (Fig. 5C). Axon C follows a more lateral path in the connective than D, M and K. As in the woodlouse, each of these axons takes an initial path around the junctions between central somata, rather than growing over their dorsal or ventral surfaces. Occasionally, axons partially enwrap central somata, resulting in the formation of short branches which run in an opposite direction to the main course of the axon (e.g. see axon C in Fig. 6C). Such branches are seen less frequently later in development.

A major difference between the crayfish and the woodlouse lies in the initial formation of the anterior commissure. An unpaired cell with an anteriorly projecting axon which bifurcates at the midline is present in the crayfish. Whilst we have tentatively identified this cell as the equivalent of the woodlouse S neuron, it is not the pioneer of the anterior commissure, as its axon grows out relatively late in embryogenesis. Axon X, which grows up the ipsilateral longitudinal connective from a posteriorly located cell, enters the anterior commissure at an early stage of its development (Fig. 5C) and may be the pioneer of this axon tract. To date, we have not identified a crayfish equivalent to the woodlouse Z neuron, which pioneers the lateral part of the anterior commissure.

The posterior commissure in the crayfish is apparently pioneered by neuron F. The axon from this neuron grows around the lateral and anterior faces of cell E (Fig. 5C) before growing directly towards the midline. On reaching the contralateral longitudinal connective, axon F grows both anteriorly and posteriorly. The posterior commissure forms after the anterior commissure.

We have injected a number of crayfish dorsal neurons in the region posterior to the posterior commissure (Fig. 10A). One of these has an axon morphology similar to the woodlouse neuron O; it grows out relatively late in development

(around 60%). An additional neuron, which we have called E′, lies just medial to neuron E and has a similar axon morphology; its axon grows out at around 55%. Neuron N ($n$=5) is an interneuron: it axon grows anteriorly and laterally at around 60% of development. On reaching the ipsilateral connective it turns posteriorly, remaining within the tract. Short anteriorly directed branches are also present (Fig. 10A). Two crayfish neurons in this posterior group have no apparent woodlouse equivalents: neuron X, described above, and neuron H (Fig. 10A, $n$=2), which has a similar axon morphology to neuron X, except that it grows posteriorly down the contralateral connective, rather than anteriorly.

We have also stained a number of dorsal neurons, in addition to the pioneering neurons C, D and K, which lie within the central rectangle bounded by the connectives and the commissures (Fig. 10B). Neuron Q, like its woodlouse counterpart, initially lies in the dorsal-most layer adjacent to D and K, but is displaced to a ventral position shortly after the onset of axonogenesis. Its axon morphology differs from the woodlouse neuron Q in that it grows posteriorly, rather than anteriorly, in the contralateral connective. Another cell, called Q′, lies ventral and medial to Q and has a similar axon morphology to the woodlouse Q neuron. Neuron J occupies a lateral position in the ganglion and its axon follows a direct course laterally into the segmental nerve. Neuron Y lies medial to J and has a similar axon morphology to this neuron.

One particularly significant crayfish neuron in the central rectangle group which has no apparent woodlouse equivalent is cell V: this neuron has an axon morphology like the insect RP1/3/4/5 neurons, running contralaterally in the anterior commissure, then down the connective and out the intersegmental nerve (Figs 9, 10B).

The final group of neurons examined in the crayfish embryo was the set of dorsal cells lying anterior to the anterior commissure. Only one of these cells, A, has an obvious woodlouse equivalent (Fig. 8B). The other three neurons filled have axon morphologies unlike any of the woodlouse neurons in this region. One is a motorneuron, projecting an axon out the ipsilateral segmental nerve. The other two send an axon across the anterior commissure into the contralateral connective: one of these axons grows anteriorly in the connective; the other branches within the contralateral neuropile (Fig. 8B).

The axon morphologies of the embryonic crayfish neurons examined in this study are summarized in schematic form in Fig. 8B.

## DISCUSSION

### A comparison of early axon growth in the Crustacea and Insecta

This study set out to address the hypothesis advanced by Thomas et al. (1984) that ‘all arthropod nervous systems seem to be constructed using the same embryonic plan’, a conclusion drawn from their observation that a set of 8 pioneering central neurons, strongly conserved in insect evolution, is present unchanged in the embryo of the crayfish *Procambarus*. The neurons in question were aCC, pCC, RP2, MP1, dMP2, vMP2, RP1 and MP4. We sought to establish whether the pattern of axon growth from the neurons that pioneer central pathways in the embryo of the isopod *Porcellio scaber* and the decapod *Cherax destructor*, is identical to that seen in insect embryos, as would be expected if the Thomas et al. claim for strict conservatism of these developmental processes is correct. Differences in the pattern of axon growth are to be expected at later developmental stages when any two species are compared: the central issue here is whether the earliest phase of axon growth is identical in the Crustacea and the Insecta. To identify this stage with absolute certainty, electron-microscopic techniques would be required. However, our use of a combination of light microscopic techniques (anti-HRP and phalloidin staining, Nomarski optics, intracellular injection of dorsal neurons), gives us confidence that we have identified the central pioneering axons in the woodlouse and crayfish CNS.

Fig. 8A,B summarizes our present findings of the axon morphology of central neurons in the woodlouse and crayfish embryos, respectively, while Fig. 11 shows the morphology of neurons in similar positions in the *Drosophila* and grasshopper embryos, drawn from Raper et al. (1983); Goodman et al. (1984); Thomas et al. (1984); Jacobs and Goodman (1989); and Sink and Whitington (1991). To assess whether a neuron in one species has a counterpart in another species, we have used two criteria.

(a) A common soma position with respect to landmarks such as axon tracts, segmental boundaries and surrounding cells in the two species. We have not applied this criterion rigidly, since soma position can change significantly in a given species over a short developmental period.

(b) A similar axon morphology in the two species. Again, we have allowed for some variation in this feature: two axons were judged to have a similar morphology if their overall trajectory was the same.

Applying these criteria, it becomes immediately apparent that the pattern of axon growth from pioneering central neurons in the woodlouse and crayfish embryos is not identical to the insect pattern. The following differences have been observed.

(a) Neither the woodlouse nor crayfish embryo appears to possess neurons in a corresponding position and with a similar axon morphology to the insect neurons MP1 and dMP2.

(b) The woodlouse embryo lacks a neuron like the insect RP1/3/4 neurons.

(c) A number of neurons in the crustacean embryos do not appear to have insect equivalents. These include the crayfish neurons N, Q, H, J, O, X, Y and the woodlouse neurons N, I, T and R.

(d) The formation of the posterior commissure appears to proceed in a different manner in the two groups. The pioneer of the posterior commissure in insects, neuron Q, lies lateral to the longitudinal connectives, whereas in the crustaceans, it lies medial to the connectives, albeit at a similar position to Q in the anteroposterior axis.

Some of these differences concern neurons that send out axons relatively late in development, well after the pioneering phase of central axon growth. Other differences are

not consistent across the two crustacean species examined (e.g. the crayfish possesses a neuron, V, with a similar axon morphology to the insect RP1/3/4 neurons, whereas the woodlouse appears to lack such a neuron). As such, they may not be relevant to the issue of identity of early neural development in the Insecta and Crustacea. When these differences are excluded, one is left with a relatively small group of differences between the two crustaceans and the insects.

This list of differences should be weighed against the many points of similarity in the spatiotemporal pattern of pioneering axon growth we observed in these two groups of arthropods. These similarities include the following.

(a) The crustacean neuron M, like the insect neuron pCC, which lies in a similar position, sends an axon anteriorly, pioneering the longitudinal connectives. The initial behaviour of M's axon as it grows around the soma of neuron E, is strikingly similar to that shown by pCC's axon, as it grows around the soma of aCC.

(b) The crustacean neurons E and E′, like the insect neurons aCC and U, which lie in equivalent positions, pioneer the intersegmental nerve.

(c) The crustacean neurons D and K, like the insect neuron vMP2, send an axon ipsilaterally and anteriorly up the connective. Like vMP2, D and K fasciculate with another axon (M in crustaceans, pCC in insects) to pioneer the intersegmental link in the connectives. In both cases these pioneering axons grow along the medial edge of the future connective.

(d) Neuron S in the woodlouse and crayfish, like the insect neuron MP4 and the DUM/VUM neurons, sends an axon anteriorly along the midline and bifurcates at the anterior commissure.

(e) The crustacean neuron C, like the insect neuron RP2, sends an axon out the ipsilateral intersegmental nerve in the next most anterior segment and is an early contributor to that nerve.

(f) The crustacean neuron A, like the locust neurons P1/P2, sends an axon posteriorly down the ipsilateral connective.

(g) The crayfish neuron Q′ and the woodlouse neuron Q, like the insect neuron A, sends an axon across the anterior commissure then anteriorly along the connective.

The question arises as to whether the neurons in the two groups of arthropods with similar axon morphologies are homologous, i.e. whether the similarities stem from the two groups sharing a common ancestral program for neural development or whether they represent evolutionary convergence. We submit that the extent of similarity in axon growth between the insects and the crustaceans examined here presents a strong argument for homology. However, sufficient differences were observed between these groups to suggest that comparisons of the spatiotemporal sequence of axon growth alone will not enable this issue to be resolved. Rather, it will be necessary to examine a suite of characteristics of neurons in the two groups, such as their lineage, neurotransmitter synthesis, or expression of homologous genes in the two groups. This information may also provide insights into the developmental origins of the differences that are seen between the crustaceans and the insects.

How can our finding of differences between the woodlouse and freshwater crayfish *Cherax* and the insects in the early development of central axon pathways be reconciled with the report by Thomas et al. (1984) that the population of pioneering central neurons in the crayfish *Procambarus* and the insects is identical? As no figures of axon morphology of *Procambarus* neurons appear in the Thomas et al. report, this question cannot be definitely answered. One possibility is that the crayfish used in the Thomas et al. study, *Procambarus*, has a different pattern of early axon development to the crustaceans examined in our study. Alternatively, Thomas et al. may not have recognized differences between the crayfish and the insects, perhaps because they sampled axon morphologies of a subset of central neurons which happen to be similar in the two species.

### A comparison of early axon growth in the crustaceans *Porcellio scaber* and *Cherax destructor*

As well as shedding light on changes in the program for early central axon growth across two major arthropod groups, the Insecta and the Crustacea, our study provides information on how these processes have been altered within one of these groups, the Crustacea. The woodlouse and the crayfish are not closely related, belonging to different Superorders, Peracarida and Eucarida, respectively, within the Subclass Eumalocostraca. Nonetheless, there are strong similarities in the pattern of axon growth from pioneering neurons in these two species and there is little doubt that several of the neurons are homologous. For example, the path followed by the axons of neurons A, C, D, E, F, J, K, M, O and S is very similar in the two species. Species differences that are observed in the axon morphology of these neurons are relatively minor: the initial course of the axon may be slightly different (e.g. neurons C, D and M) or the axon morphology in one species can be derived from the equivalent cell in the other species by the addition of a single branch (e.g. the crayfish F axon grows both anteriorly and posteriorly down the connective, whereas its woodlouse equivalent only grows anteriorly). Furthermore, the relative timing of axon outgrowth is the same for a subset of these neurons, namely C, D, E, F, K and M. The longitudinal connectives, the intersegmental nerve and the posterior commissure are pioneered by corresponding axons (M/D/K/C, E and F respectively) in the two species, (although the crayfish M/D/K axons are preceded by axons from brain neurons, as discussed below).

The relative timing of axon outgrowth from neuron S does differ between these two crustaceans. In the woodlouse, neuron S sends out an axon early in development, pioneering a medial axon tract and participating in the initial formation of the anterior commissure. The crayfish equivalent of this neuron grows out much later, after the anterior commissure has been well established.

For another group of neurons, which includes N and Q, there are similarities in axon morphology in the two species, but also some differences. The axon morphology of the woodlouse N neuron is the same as its crayfish equivalent except that it sends a branch into the periphery, whereas the crayfish neuron remains within the CNS. The wood-

louse Q axon runs anteriorly in the connective whereas the crayfish Q axon runs posteriorly. The case for homology based on axon morphology is much weaker for these neurons.

Another set of neurons appears to be specific to one or the other crustaceans. The anterior commissure in the crayfish is apparently pioneered by an axon, X, which we have not identified in the woodlouse and conversely, we have not found a crayfish equivalent to the woodlouse neuron Z, the other cell involved in the establishment of the anterior commissure in the woodlouse. Woodlouse counterparts to the crayfish neurons H, V, Y and E′ have not been located, while we have not found crayfish neurons with axon morphologies like the woodlouse neurons L, I and R. Homologues to each of these neurons may exist in the other species but in more ventral positions of the ganglion, which we have not explored. Alternatively, these neurons may have been lost during the course of evolution (by neural death) or they may have changed their axonal morphology. Additional studies of cell lineage or patterns of expression of neurotransmitters and identified, homologous gene products may help to decide between these possibilities.

Finally, a substantial difference exists between the woodlouse and the crayfish in the source of the longitudinal pioneering axons: in the woodlouse this pathway is pioneered by segmental neurons (specifically, axons M/C/D/K), whereas the first longitudinal axons in the crayfish arise from neurons located in the brain. This difference may reflect the relative precocity of formation of head segments with respect to more posterior body segments in the crayfish (Scholtz, 1992). Posteriorly coursing axons from brain neurons may appear in the longitudinal connectives relatively later in the woodlouse, after segmental neurons have already begun axonogenesis. This may represent another example of heterochrony, the well-documented phenomenon of change in timing of equivalent developmental events in related groups of organisms (Raff and Kaufman, 1983).

### A comparison of early axon growth in the Crustacea, Insecta and Myriapoda

In our earlier study of neural development in the centipede *Ethmostigmus rubripes* (Whitington et al., 1991), we reported that the longitudinal connectives are pioneered, not, as in insects, by segmental neurons, but by the posteriorly directed growth of axons originating from neurons located in the brain. Furthermore, the pattern of segmental neurons involved in later axon growth bears no obvious similarities to that found in insects; many more cells are present in the CNS at the time of segmental axon formation in centipedes than in insects and the contributing neurons are much more widely separated. In contrast, many of the pioneering neurons in the woodlouse and crayfish embryos have similar axon morphologies to insect neurons. In addition, central pathways in these crustaceans are pioneered when there is a relatively small population of neurons and the distances between the neurons involved are short.

Overall, a comparison of early neural development in the woodlouse and the insects reveals many more similarities than a comparison of the same processes in the centipede and the insects. (Although, the fact that the longitudinal connectives in the crayfish are pioneered by axons from brain neurons, rather than segmental neurons, suggests that this particular difference between insects and myriapods should not be taken as an indication of evolutionary divergence). This is a surprising finding, given that insects are widely held to have evolved from a myriapod-like ancestor and that, compared to the myriapods, the Crustacea are relatively distantly removed from the insects. On the other hand, a recent study of 12S rRNA sequences in a range of arthropods (Ballard et al., 1992) indicates that Insecta and Crustacea are more closely related than either group is to the Myriapoda.

We thank Eldon Ball, Nipam Patel, Gerhard Scholtz and David Sandeman for their criticisms on this manuscript and Zoltan Enoch for photographic assistance.

## REFERENCES

**Ballard, J. W. O., Olsen, G. J., Faith, D. P., Odgers, W. A., Rowell, D. M. and Atkinson, P. W.** (1992). Evidence from 12s ribosomal RNA sequences that onychophorans are modified arthropods. *Science* **258**, 1345-1348.

**Dohle, W. and Scholtz, G.** (1988). Clonal analysis of the crustacean segment: the discordance between genealogical and segmental borders. *Development* **Supplement104**, 147-160.

**Goodman, C. S., Bastiani, M. J., Doe, C. Q., duLac, S., Helfand, S. L., Kuwada, J. Y. and Thomas, J. B.** (1984). Cell recognition during neuronal development. *Science* **225**, 1271-1279.

**Gould, S. J.** (1977). *Ontogeny and Phylogeny* Harvard, USA: Belknap Press.

**Harvey, J. J. and Raff, R. A.** (1990). Evolutionary change in the process of dorsoventral axis determination in the direct developing sea urchin, *Heliocidaris erythrogramma*. *Dev. Biol.* **141**, 55-69.

**Jacobs, J. R. and Goodman, C. S.** (1989). Embryonic development of axon pathways in the *Drosophila* CNS. 2. Behavior of pioneer growth cones. *J. Neurosci.* **9**, 2412-2422.

**Patel, N. H., Poole, S. J., Coleman, K. G., Goodman, C. S., Ellis, M. C., Martinblanco, E. and Kornberg, T. B.** (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

**Raff, R. and Kaufman, T.** (1983). *Embryos, Genes and Evolution.* New York: MacMillan.

**Raper, J. A., Bastiani, M. J. and Goodman, C. S.** (1983). Pathfinding by neuronal growth cones in grasshopper embryos: II selective fasciculation onto specific axonal pathways. *J. Neurosci.* **3**, 31-41.

**Sandeman, R. and Sandeman, D.** (1991). Stages in the development of the fresh-water crayfish *Cherax destructor*. *Roux's Arch. Dev. Biol.* **200**, 27-37.

**Scholtz, G.** (1992). Cell lineage studies in the crayfish *Cherax destructor* (Crustacea, Decapoda) - germ band formation, segmentation, and early neurogenesis. *Roux's Arch. Dev. Biol.* **202**, 36-48.

**Sink, H. and Whitington, P. M.** (1991). Location and connectivity of abdominal motoneurons in the embryo and larva of *Drosophila melanogaster*. *J. Neurobiol.* **22**, 298-311.

**Thomas, J. B., Bastiani, M. J., Bate, M. and Goodman, C. S.** (1984). From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* **310**, 203-207.

**Whitington, P. M., Meier, T. and King, P.** (1991). Segmentation, neurogenesis and formation of early axonal pathways in the centipede, *Ethmostigmus rubripes* (Brandt). *Roux's Arch. Dev. Biol.* **199**, 349-363.

**Wray, G. A. and Raff, R. A.** (1990). Novel origins of lineage founder cells in the direct developing sea urchin, *Heliocidaris erthyrogramma*. *Dev. Biol.* **141**, 41-54.

(Accepted 19 March 1993)