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Normal and abnormal development of an identified leech motor neuron

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SUMMARY

In embryonic and mature leeches, the identified L motor neuron, which innervates the longitudinal muscles of the contralateral half body segment, can be identified by the location and relatively large size of its cell body. Here the morphological and physiological development of the L motor neuron has been investigated by intracellular recording and dye-filling techniques in normal and abnormal embryonic leeches. Normally the L motor neuron growth cone projects from the cell body at about the same time as from many other neurons located in the lateral part of the ganglion, including the P mechanosensory neurons. The L motor axon, like many other leech axons, projects directly into the appropriate pathway. The L motor neuron does not initially extend an excessive number of axons followed by elimination of the inappropriate ones. Its growth cone is tapered and relatively free of filopodia and grows out of the ganglion in the contralateral posterior nerve behind the growth cone of the primary peripheral axon of the dorsal P mechanosensory cell, which is one of the earliest axons in the posterior root.

Occasionally the bilateral halves of the germinal plate fail to fuse resulting in an embryo with separated but intact half ganglia, body wall, and skin. In such embryos the L motor neuron axons cannot grow out the contralateral posterior nerve since it is not available. Instead they grow out a variety of ipsilateral nerves and/or connective tracts. The P mechanosensory cells, which normally grow out of the ganglion in specific ipsilateral nerves, extend their axons along their normal pathways. In these abnormal embryos the L motor neurons did not preferentially grow into the ipsilateral posterior nerve, normally the pathway taken by the bilateral homologue and the nerve most similar to the L motor neuron's normal pathway. The failure of these L neurons to either consistently choose or avoid the ipsilateral posterior root suggests that the bilateral homologues ignore one another's pathfinding cues or that such cues are missing or changed in these embryos. The axons of the P neurons, however, appear to require no cues or interactions with contralateral structures or cells for normal development.

INTRODUCTION

The analysis of axon outgrowth by identified neurons in accessible embryonic nervous systems has demonstrated neuronal specificity and the orderliness of growth cone behaviour. For example, early in the embryogenesis of the grasshopper nervous system, when the cellular landscape is simple enough to identify many if not all of the elements, it has been demonstrated that an identified

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growth cone finds its target by making a series of relatively simple choices (Goodman, Raper, Ho & Chang, 1982; Ho & Goodman, 1982; Bentley & Keshishian, 1982; Raper, Bastiani & Goodman, 1983*a*,*b*). Thus the notion of neuronal specificity has been simplified and refined into a set of well-defined decisions made by a particular growth cone. The basis for these decisions has been hypothesized to include environmental cues such as 'landmark' cells (Goodman *et al.* 1982; Ho & Goodman, 1982) or 'guidepost' cells (Bentley & Keshishian, 1982) and 'labelled' pathways (Raper *et al.* 1983*a*,*b*).

Likewise in the embryonic leech, most identified neurons, exhibit orderly growth and target specificity (Kuwada, 1982; Kuwada & Kramer, 1983; Kramer & Kuwada, 1983). Here the development of the identified L motor neuron is described in normal embryos and in embryos in which the bilateral halves of the germinal plate have failed to fuse resulting in embryos consisting of two separated halves. These abnormal embryos are particularly interesting because the L axon normally leaves the CNS in a specific nerve contralateral to its cell body to innervate body wall muscles. Since the contralateral half of the embryo is not available in these abnormal embryos, we can assess the growth of an axon when some of its normal cues are inaccessible.

METHODS

Embryos of the leech *Haementaria ghilianii* were supplied by a laboratory breeding colony. Details of the growth, reproduction, and care of adult *H. ghilianii* are given in Sawyer, LePont, Stuart & Kramer (1981) and of their embryos in Kuwada & Kramer (1983). Kuwada & Kramer (1983) also fully describe the dissection of the embryos and the procedures followed to reveal the physiology and anatomy of embryonic leech neurons. In short, embryos were dissected, pinned out in a recording chamber, and placed on a compound microscope. Embryonic neurons were visualized in the living preparation with Nomarski optics and their physiology and morphology analysed via intracellular recordings and by filling them with the fluorescent dye, Lucifer Yellow (Stewart, 1978).

A set of external morphological characteristics correlated with each day of development at 27 °C (unpublished data) was used to stage embryos. A brief description of the embryos at the stages relevant to this report and the general features of the nervous system during embryogenesis can be found in Kuwada & Kramer (1983). The stage of an embryo was designated by the stage number followed by, in parentheses, the ratio of the number of days elapsed since the beginning of the stage to the total number of days in the stage. A stage-10(1/5) embryo has completed the first of 5 days in stage 10. The morphologies of the embryonic L motor neurons are based on three to five dye fills at each time point. Slides of the filled cells were photographed and the structure of each cell was reconstructed from the slides.

RESULTS

Morphological development of the L motor neuron

The leech comprises 32 body segments including 21 essentially identical midbody segments. The central nervous system reflects this structure and is made up of a chain of ganglia, one per segment, linked to each other by connectives and to the periphery via a well-defined set of nerves (Muller, Nicholls & Stent, 1981). The L motor neuron is an excitatory motor neuron which innervates the ventral and dorsal longitudinal muscles of the half segment contralateral to its cell body (Stuart, 1970; Kramer & Goldman, 1981). It has a large cell body (40–50 μm in diameter) from which an axon projects straight across the midline of the ganglion, turns posteriorly and laterally towards the posterior nerve root, leaves the ganglion in the posterior nerve, and courses through the body wall in the posterior posterior (PP) nerve in *H. ghilianii* (Kramer & Goldman, 1981; Fig. 1). In the ganglion various neuropilar processes come off the axon including a

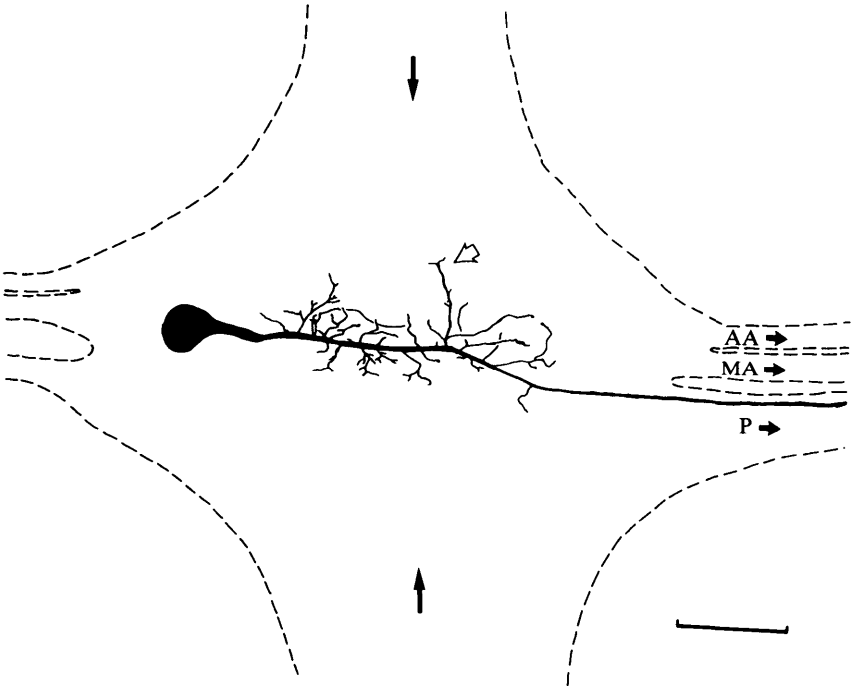

Fig. 1. The morphology of an L motor neuron from a midbody ganglion in a small adult leech. Dashed lines denote the outline of the ganglion and nerves; anterior is up; black arrows indicate the midline; open arrow points to the characteristic, anteriorly directed neuropilar process; AA is the anterior anterior nerve, MA is the medial anterior nerve, and P the posterior nerve. The posterior nerve branches relatively near the ganglion to give rise to the posterior posterior nerve and the dorsal posterior nerve. Scale 100 μm.

characteristic, long anteriorly directed process extended from the axon where it turns toward the posterior nerve root.

The L motor neuron is readily found in the adult due to its relatively large cell body which is reliably located on the lateral part of the dorsal surface of the ganglion just posterior to the MA nerve root and medial to the large laterally located ventral P neuron (Fig. 2A). In embryonic ganglia a relatively large soma, which presumably is the L motor neuron, can be found in the same location (Fig. 2B).

The embryonic L motor neuron grows a medially directed growth cone which reaches or crosses the midline by stage 9(1·5/4) to 9(2/4) (Fig. 3A). Thus, the L motor neuron first extends a growth cone at about the same time as many laterally located neurons and within a day of the identifiable mechanosensory P neurons (Kuwada & Kramer, 1983). Approximately one half day later, the growth cone has clearly turned towards the nascent posterior nerve root and, although still in the ganglion, can be found near the entrance to the posterior nerve (Fig. 3B). At this time fine processes, which may develop into neuropilar processes, can be seen extending from the axon. Also the L growth cone is tapered with relatively few filopodia as it leaves the ganglion (Fig. 4B), rather than broad with many filopodia like the dorsal P peripheral growth cone (Kuwada & Kramer, 1983; Fig. 4C). In the next half to one day the growth cone has left the ganglion in the posterior nerve root and grown laterally for about 80 μm

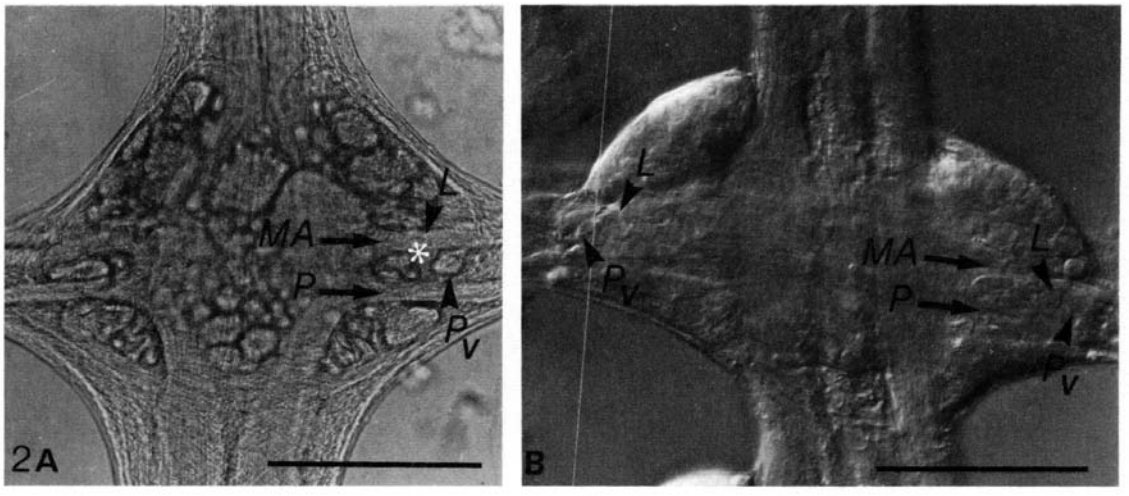

Fig. 2. Characteristic location of the L cell body. (A) light micrograph of a midbody ganglion from a young adult. The L and ventral P cell bodies on the left side of the ganglion are out of focus. Scale 250 μm. (B) Nomarski micrograph of a midbody ganglion from a stage-10(0/5) embryo. This micrograph was composed from two photographs taken at slightly different focal planes. Scale 50 μm. L denotes the L cell body, P_V the ventral P mechanosensory cell body, MA the medial anterior nerve, and P is the posterior nerve. An asterisk is within the L cell body to clarify its location in panel *A*. Anterior is up.

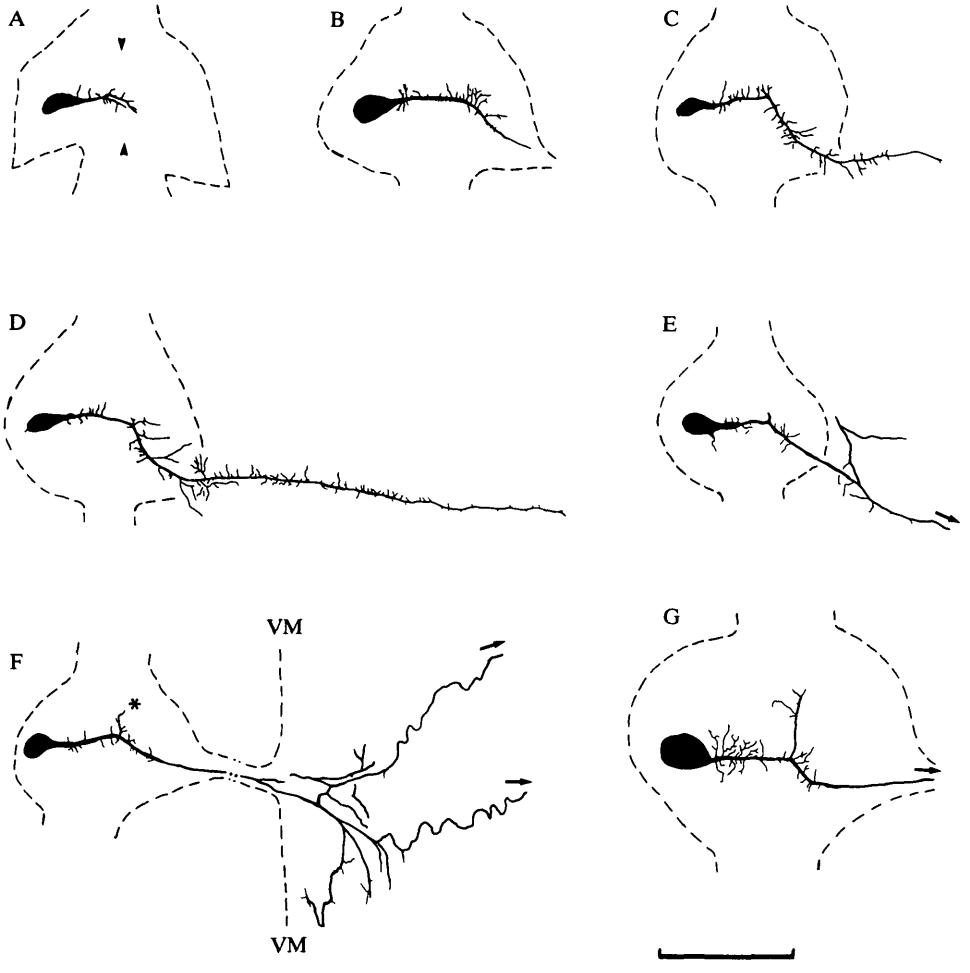

Fig. 3. The morphology of developing L motor neurons from midbody ganglia. (A) stage 9(1·5–2/4). Arrowheads indicate the midline. The ganglion is outlined by dashed lines. Anterior is up. (B) stage 9(2–2·5/4). (C) stage 9(2·5–3/4). The growth cone was in the vicinity of the developing nephridia. (D) stage 9(3–3·5/4). This axon had grown to the future lateral edge. (E) stage 10(1/5). The arrow signifies that the axon continues beyond the reconstruction. (F) stage 10(3/5). In this preparation the body wall was halved longitudinally by cutting along the dorsal and ventral midlines. VM indicates the ventral midline of the body wall. The dots denote that the nerve and axon are continuous. An asterisk marks the anteriorly directed neuropilar process. (G) stage 11(2/20). In C–G the L axon leaves the ganglion via the posterior root. Scale 100 μm.

to the level of the nascent nephridia without branching in the developing body wall (Fig. 3C). The position of the nephridia in relation to the ganglion can be seen in Fig. 6A. By another half to one day the L growth cone was often found in the vicinity of the future lateral edge, which demarcates the ventral from

dorsal halves in the adult leech body (Fig. 3D). The future lateral edge occurs approximately halfway between the edge of the ganglion and the lateral border of the germinal plate (Kuwada & Kramer, 1983). Soon after the axon leaves the ganglion, its pathway (presumably the PP nerve pathway) is deep in the developing body wall at the level of the developing longitudinal muscles (data not shown). Lateral filopodia are present on the peripheral axon of the L motor neuron at this time (Fig. 3D).

By stage 10(1/5) the peripheral axon has branched to give rise to an anteriorly directed longitudinal branch near the ganglion and a lateral branch off the longitudinal branch (Fig. 3E). Two days later there are more branches of the peripheral axon including posterior branches, and the characteristic, anteriorly directed neuropilar process is well formed (Fig. 3F). Finally, the central processes of the embryonic L motor neuron are comparable to those seen in the adult leech by early stage 11 (Fig. 3G).

As mentioned above, the L motor neuron extends a growth cone within a day

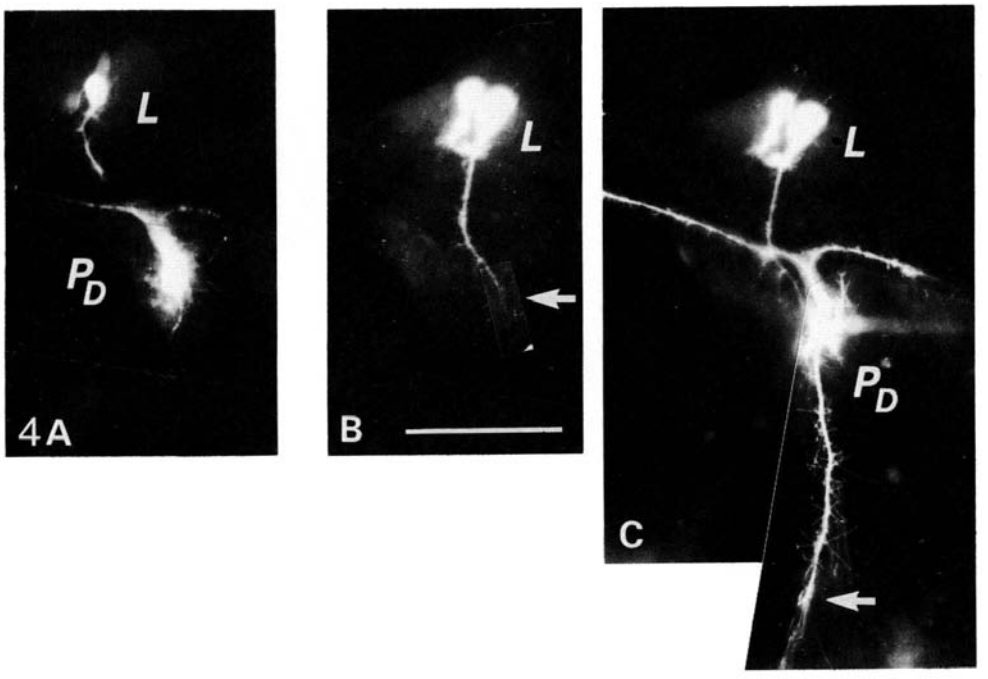

Fig. 4. The L axon grows out of the CNS after the dorsal P primary peripheral axon. (A) photograph of a Lucifer-Yellow-filled L motor neuron and a contralateral dorsal P neuron from ganglion 12 in a stage-9 (2/4) embryo. L denotes the L motor neuron and P_D the dorsal P neuron. Anterior is to the left. (B) photograph of a Lucifer-Yellow-filled L motor neuron from ganglion 9 in a stage-9(2·5–3/4) embryo. An unidentified neuron anterior to the L motor neuron was also filled in this ganglion. (C) photograph of the contralateral P_D neuron filled with Lucifer Yellow and the same L motor neuron from (B). Arrow in (B) signifies the growth cone of the L motor neuron and the arrow in (C) denotes the growth cone of the dorsal P neuron. Scale 100 μm.

of the dorsal P neuron and both neurons exit the ganglion via the posterior nerve. However, the L motor neuron growth cone leaves the ganglion after the dorsal P neuron's primary peripheral axon, which is one of the earliest peripheral axons in the leech nervous system (Kuwada, 1982; Kuwada & Kramer, 1983). The dorsal P neuron has already extended numerous peripheral neurites, one of which will develop into the primary peripheral axon (Kuwada & Kramer, 1983), by the time the L motor neuron growth cone reaches the midline (Fig. 4A). Later when the L motor neuron axon is about to leave the ganglion, the dorsal P neuron has an established primary peripheral axon (Fig. 4B, C) whose growth cone can be in the vicinity of the nephridia and the future lateral edge. In older embryos the L axon diverges from the dorsal P peripheral axon; the former grows deep in the developing body wall in the PP pathway and the latter in the superficial dorsal posterior (DP) pathway (data not shown).

Physiological development of the L motor neuron

In adult leeches intrasomatically recorded action potentials of L motor neurons are typically 10 mV or less and probably represent non-invading axon action potentials (Stuart, 1970; Kramer & Goldman, 1981). Likewise in the embryo intrasomatically recorded action potentials are small and can be elicited via current injection by stage 10(1/5) (Fig. 5A). Note that by this time the L motor neuron has a well-developed peripheral axon which has already begun to branch in the body wall (see above section).

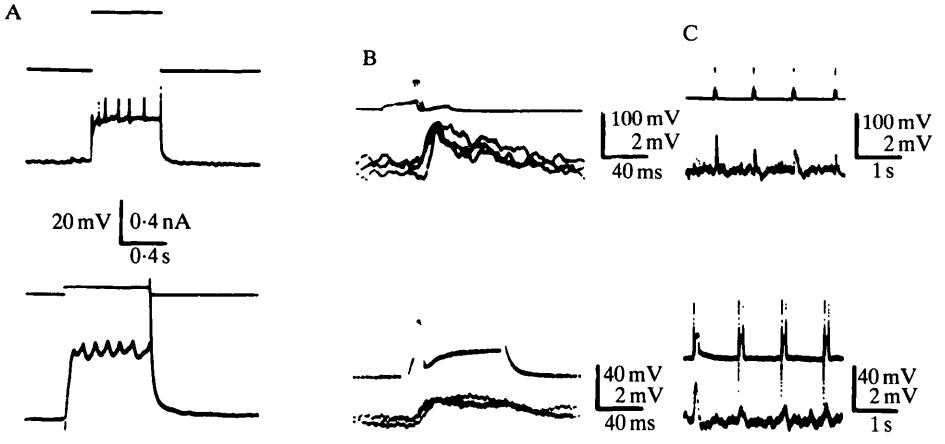

Fig. 5. The physiology of adult and embryonic L motor neurons. (A) action potentials elicited by current injection into the soma of an adult (top panel) and stage-10(1/5) (bottom panel) L motor neuron. In each panel the top trace is the current record and the bottom trace is the voltage record. (B) synaptic potentials recorded in the L motor neuron following action potentials evoked via current injection in the contralateral dorsal P neuron in an adult (top panel) and stage-11(4/20) embryo (bottom panel). In each panel three superimposed traces are shown with the top trace being the dorsal P neuron and the bottom trace the L motor neuron. (C) depression of the synaptic potentials when stimulated at 1 Hz from the same neurons as shown in (B). The top panel are records from the adult and the bottom panel are from the embryo.

The L motor neuron receives monosynaptic chemical and electrical input from a variety of identified neurons, including the mechanosensory P neurons (Nicholls & Purves, 1970; Kramer, 1981). Furthermore, the synapse between the P neurons and the L motor neuron exhibits short-term plasticity in the leech *Hirudo medicinalis* (Nicholls & Purves, 1972). In adult and embryonic *H. ghilianii* action potentials elicited from P neurons via current injection are followed by short, constant latency excitatory postsynaptic potentials (PSP's) (Fig. 5B), which will decrease in amplitude when a P neuron is repetitively stimulated at a rate of 1 Hz (Fig. 5C). The PSP's are excitatory since they can sometimes give rise to action potentials in the L motor neuron (data not shown). Although it is not known when these synapses become functional, they have been observed as early as stage 11(0/20) (data not shown).

Morphology of L motor neurons in abnormal embryos

The germinal plate of a leech embryo is formed by columns of blast cells produced by asymmetric divisions of a set of five teloblasts on each side of the midline (Weisblat, Sawyer & Stent, 1978; Weisblat, Harper, Stent & Sawyer, 1980; Weisblat, Zackson, Blair & Young, 1980). Normally the columns of progeny from the two bilateral sets of teloblasts coalesce at the midline to form the germinal plate. Occasionally, however, the two sets of progeny fail to coalesce or do so only in the anterior-most region. This produces late stage-9 to early stage-10 embryos in which the two bilateral halves are not fused or are only fused at the anterior end. Each bilateral half-embryo appears normal and contains developing body wall (including muscle), skin, nephridia, and half of a

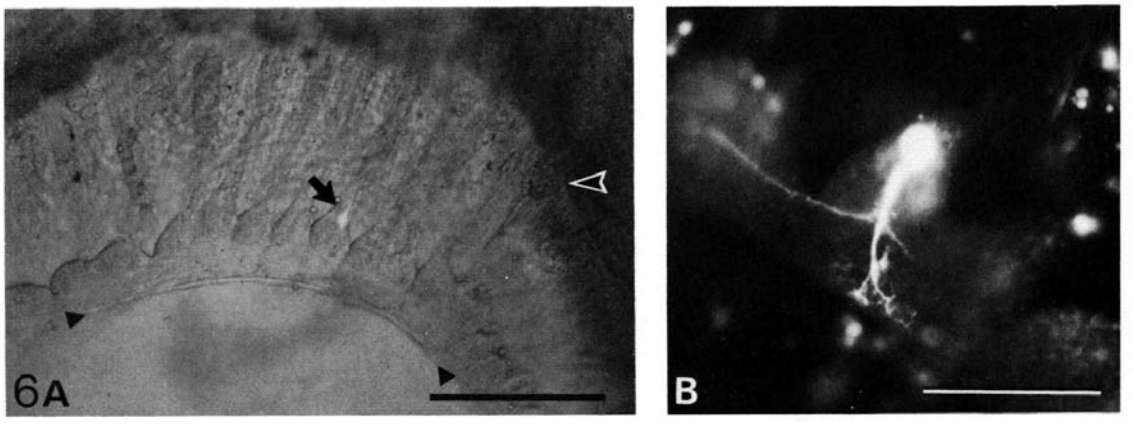

Fig. 6. Photographs of abnormal, uncoalesced embryos. Anterior is to the left. (A) a stage-10(0/5) embryo. Arrow points to a Lucifer-Yellow-filled dorsal P neuron, the triangles denote the ventral midline and the arrowhead points to one of the developing nephridia. The peripheral nerves are out of focus and difficult to see at this magnification. The outlines of the ganglia appear somewhat deformed and the connectives are not stretched because the abnormal embryos are difficult to pin out. Scale 250 μm. (B) a presumptive L motor neuron filled with Lucifer Yellow in ganglion 9 of a stage-9(3/4) uncoalesced embryo. This neuron did not exit the nervous system. Scale 50 μm.

nervous system (including half ganglia, one set of connective tracts, and ipsilateral peripheral nerves) (Fig. 6). In these half-ganglia the relative sizes and arrangement of neurons appeared normal, e.g., large neuronal somata could be found in the normal positions for the two P neurons and the L motor neuron. In

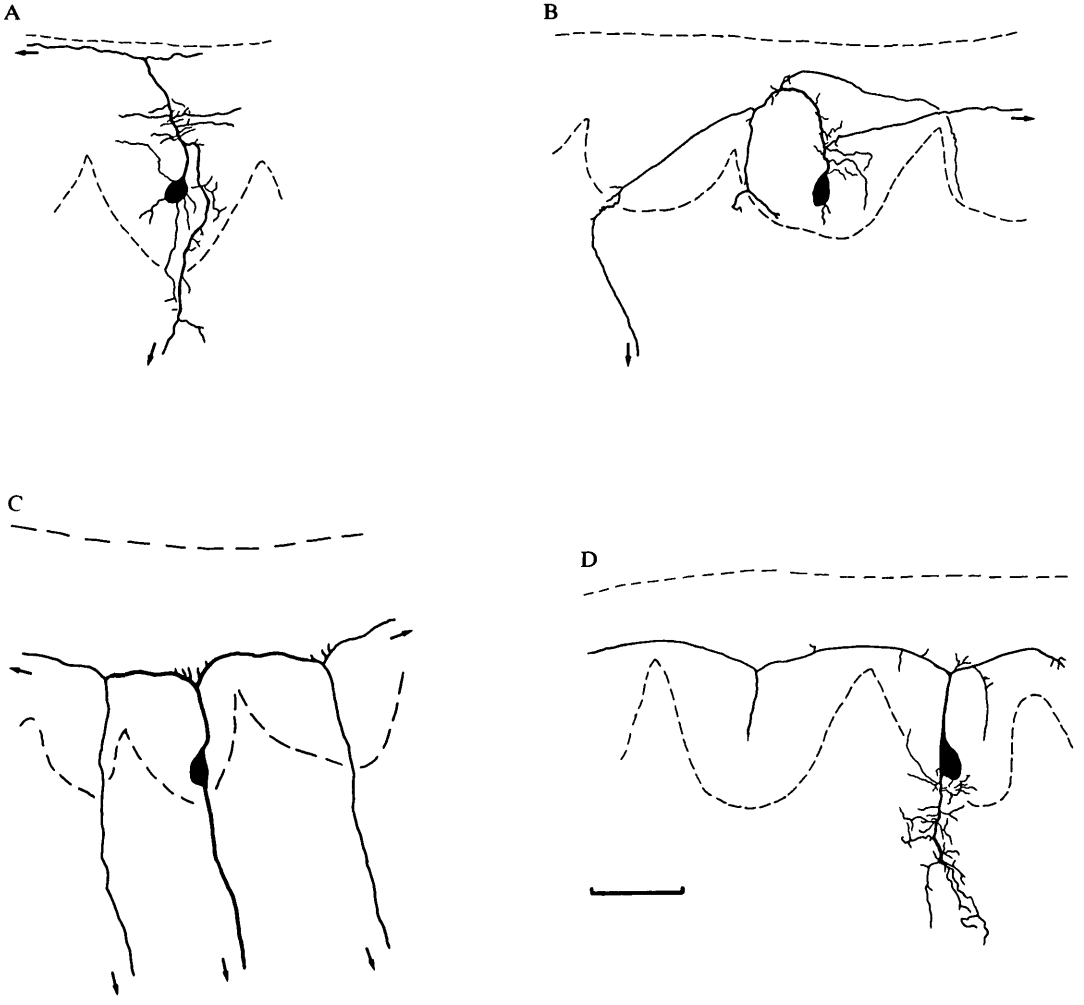

Fig. 7. Examples of neurons in uncoalesced embryos. Anterior is to the left. (A) a presumptive L motor neuron from ganglion 11 in a stage-9(3/4) embryo. The abnormal ipsilateral peripheral axon left the ganglion via the posterior nerve. Arrows indicate that the axons continue beyond the limits of the reconstruction. (B) a presumptive L motor neuron from ganglion 7 in a stage-9(3/4) embryo. This L motor neuron left the ganglion containing its cell body in an abnormal anterior pathway and in a pathway from the adjacent anterior ganglion. (C) a dorsal P neuron from ganglion 8 in a stage-10(0/5) embryo. All the peripheral axons left their respective ganglia in the posterior nerve as in normal embryos. (D) a ventral P neuron from ganglion 13 in a stage-9(3/4) embryo. The peripheral axon left the ganglion in the medial anterior (MA) nerve as in normal embryos. Scale 50 μm.

these cases the identity of a neuron was presumed to be that of the neuron occupying that same position in normal ganglia. It is possible that one neuron of a dorsal cluster of motor neurons, which also project axons out the CNS in contralateral nerves, may have been mistaken for the L motor neuron, but their cell bodies tend to be both smaller and more medial than the L motor neuron in normal embryos. Two large neurons in the vicinity of the L motor neuron, the ventral P and Leydig neurons have ipsilateral axons making it unlikely that they would be mistaken for the L motor neuron.

In four stage-9(3/4) to -10(1/5) split embryos, ten L motor neurons were filled with Lucifer Yellow dye. Recall that by this time the L motor neurons normally have extended a peripheral axon out the *contralateral* posterior nerve (see Fig. 3C, D), which is not available to L motor neurons in these abnormal embryos. Instead their axons course to the midline and often branch abnormally. Axon branches can follow connective tracts but never leave the CNS (Fig. 6B) (n = 2), grow out the CNS via the *ipsilateral* posterior nerve (Fig. 7A) (n = 3), or grow out a variety of other nerves both of the ganglion containing the L soma and adjacent ganglia (Fig. 7B) (n = 5). There was no discernable pattern of axonal growth in the ten motor neurons analysed. When the aberrant L motor neurons have connective axons, they are not necessarily close to the connective axons of the P neurons (data not shown). In the same embryos the morphologies of the two P neurons are normal (Fig. 7C, D). P neurons normally have only ipsilateral peripheral axons in adult leeches (Nicholls & Baylor, 1968; Kramer & Goldman, 1981) and in embryonic leeches (Kuwada & Kramer, 1983; Kramer & Kuwada, 1983).

DISCUSSION

Normal and abnormal growth of axons

The axon of the identifiable leech L motor neuron grows directly to its target via a stereotyped pathway and assumes its mature morphology without random outgrowth of axons into every available pathway followed by elimination of the inappropriate ones. This stereotyped pattern of axonal growth has been observed with identifiable pools of chick motor neurons (Landmesser, 1978*a*,*b*; Lance-Jones & Landmesser, 1980*a*,*b*, 1981*a*,*b*), and identifiable grasshopper central and peripheral neurons (Bate, 1976; Keshishian, 1980; Bate & Grunewald, 1981; Goodman, Bate & Spitzer, 1981; Ho & Goodman, 1982; Raper *et al.* 1983*a*,*b*) as well as with other leech neurons (Kuwada, 1982; Kuwada & Kramer, 1983; Kramer & Kuwada, 1983).

The L motor neuron in abnormal embryos, which have uncoalesced bilateral halves, is deprived of its normal pathway out of the ganglion, the contralateral posterior nerve. In contrast to the regular pattern of axonal growth exhibited by a normal L motor neuron, the L motor neuron in abnormal embryos branches excessively to produce several axons and these axons grow along a number of

pathways. These include inappropriate connective tracts and ipsilateral nerves as well as the nerve its contralateral homologue would normally have taken. Thus, L motor neurons under these conditions exhibit a disorganized pattern of axonal growth and do not prefer or consistently avoid the pathway of its contralateral homologue. However, eight of the ten abnormal L motor neurons did grow axons out into the periphery via ipsilateral pathways by late stage 9 to early stage 10, and the two exceptions may have done so later on. The L axon can grow into the periphery despite gross alterations in its normal pathway. This may indicate that L motor neurons have the ability to find suitable muscle targets despite the alterations. However, this is not yet known since it is not known whether the abnormal L motor neurons can innervate the ipsilateral counterparts of their normal targets or any muscles when their normal pathways have been disrupted. Thus, it is difficult to draw conclusions concerning the nature of cues normally utilized by the L motor neuron during axonogenesis from the abnormal embryo data especially since we do not know how such cues may have been affected by the abnormal development. It is possible, for example, that the dorsal P peripheral axon, which normally leaves the ganglion via the same nerve as the L motor neuron axon but precedes the L axon, may be an important cue for the growing L growth cone. However, it is clear that despite this any dorsal P neuron is not sufficient to direct the L growth cone since in the uncoalesced embryos L axons do not preferentially grow out the ipsilateral posterior nerve.

In the same embryos the morphologies of P neurons, which normally have only ipsilateral peripheral axons, were normal. This establishes that their growth cones need not interact with cells or structures from the contralateral side for normal development.

Development of synaptic activity

Although a complete analysis of the development of synaptic activity onto the L motor neuron was not performed and the onset of synaptic activity is not known, short and constant latency synaptic input from the P neurons which decreased in amplitude with repetitive stimulation and resembled the mature synapse was observed in early stage 11. Furthermore, L motor neurons in early stage-10 embryos had already begun branching in the body wall at the level of the longitudinal muscles and were capable of producing action potentials. This correlates with the time when the mechanosensory P neurons are capable of producing action potentials (Kuwada & Kramer, 1983) and when embryos will respond behaviourly to external stimulation for the first time by contracting the whole body longitudinally (W. B. Kristan, personal communication). This may indicate that synapses made by sensory neurons, such as the P neurons, onto the L motor neuron and the neuromuscular junctions made by L motor neurons onto the longitudinal muscles may be functional early in embryogenesis.

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