

Pioneering and pathfinding by an identified neuron in the embryonic leech

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SUMMARY

Numerous investigations of pathfinding by embryonic neurons, including many leech neurons, have demonstrated that pathfinding is precise. Neurons project the correct number of growth cones which grow to their target areas by making specific choices along the way. However, one leech mechanosensory neuron, the dorsal P (P_D) neuron, is unusual in the sense that it initially projects an excessive number of growth cones. One of the growth cones will form the peripheral axon while the others are eliminated. This suggests that P_D is one of the earliest neurons to project a peripheral axon, i.e., it may pioneer a peripheral nerve, and that it searches with its multiple growth cones for an external cue which can guide it to its target area. Examination of the early P_D axon with light and electron microscopy reveals that it indeed is the first growth cone in its nerve and that it grows in contact with a large non-neuronal (DV) cell until it reaches its target area. The DV cell has a unique morphology and location: a large cell body with thin flat processes extending from the edge of the ganglion to the target area of the P_D. It is also present with its unique morphology prior to axonal outgrowth by the P_D neuron. These features suggest that the DV cell may be an attractive substrate and/or axonal guidance cue for the P_D peripheral axon and therefore for the entire peripheral nerve.

INTRODUCTION

The analysis of how neurons make specific connections with target cells has benefited from investigations of axonogenesis by identifiable embryonic neurons (for reviews see Goodman *et al.* 1984; Goodman & Bastiani, 1985). These studies demonstrated that for the most part embryonic neurons do not randomly project an excessive number of axons and then eliminate the inappropriate ones. Instead, they project the appropriate number of growth cones which grow to their targets by making a series of decisions at specific choice points. Choices appear to be made by utilizing gradients of adhesivity (Nardi, 1983; Berlot & Goodman, 1984) and by specific cellular cues. Cellular cues can be non-neuronal cells (Ho, Ball & Goodman 1983) or other neurons both in the peripheral nervous system (Ho & Goodman, 1982; Bentley & Keshishian, 1982; Bentley & Caudy, 1983; Berlot & Goodman, 1984) and in the central nervous system (CNS) (Raper, Bastiani & Goodman 1983a,b,c;

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Taghert, Bastiani, Ho & Goodman 1982; Bastiani & Goodman, 1984; Raper, Bastiani & Goodman 1984; Bastiani, Raper & Goodman 1984).

An exception to the orderly projection and growth of embryonic axons is the initial stages of the development of the primary peripheral axon of the dorsal pressure (P_D) neuron (Kuwada, 1982; Kuwada & Kramer, 1983), a mechanosensory neuron found in the CNS of the leech (Nicholls & Baylor, 1968; Kramer & Goldman, 1981). Early in embryogenesis the P_D neuron projects numerous peripherally directed growth cones. When the peripheral growth cones are still in the vicinity of the ganglion (far from the primary target area), one of them rapidly grows directly to the proper target area while the others are eliminated. This sequence of events suggests that the growth cones are sampling the environment for a cue which could direct a growth cone to the proper target area (Kuwada, 1982; Kuwada & Kramer, 1983). This phenomenon has been investigated further by marking embryonic P_D neurons and examining them with light and electron microscopic techniques. These data demonstrate that the P_D neuron is the first neuron, i.e., a pioneer neuron (Bate, 1976), in its peripheral nerve and its primary peripheral axon grows along an identifiable non-neuronal cell to get to its target area.

MATERIALS AND METHODS

Embryos of the leech *Haementaria ghilianii* were collected from a laboratory breeding colony. Descriptions of the growth, reproduction, and care of the adult leeches can be found in Sawyer, LePont, Stuart & Kramer (1981) and of the embryos in Kuwada & Kramer (1983). The dissection of the embryos and the procedures for the physiological and light level anatomical investigations of the embryonic cells have been previously enumerated by Kuwada & Kramer (1983). In short, embryos were dissected, pinned out in a recording chamber, and mounted on a fixed-stage compound microscope outfitted for Nomarski interference contrast optics and epifluorescence illumination. Cells were visualized in living embryos with Nomarski optics and subsequently penetrated with a microelectrode to record their activity and to fill them with the fluorescent dye, Lucifer Yellow (Stewart, 1978).

For the electron microscopic analysis of early axons, neurons were injected with horseradish peroxidase (HRP) (4% dissolved in 100 mM-KCl at pH 7.4) by passing 1–2 nA of depolarizing current for 5 to 15 minutes from HRP-filled microelectrodes measuring 100 to 250 megohms in resistance. Embryos were then fixed in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 100 mM-phosphate buffer at pH 7.4 for 1 h at room temperature. Following three washes (20 min each) in the phosphate buffer, embryos were incubated for 20 min in the developing solution containing 0.5 mg/ml of 3,3 diaminobenzidine, 2 mg/ml of beta-D-glucose, and 0.5 mg/ml of ammonium chloride in phosphate buffer. The HRP reaction was initiated by adding 0.5 unit of glucose oxidase per ml of developing solution and allowed to develop for a $\frac{1}{2}$ to 1 h. The embryos were washed three times for 30 min each in phosphate buffer and then postfixed in 1% osmium tetroxide in phosphate buffer on ice for 1 h. Following three more washes (10 min each) in cold phosphate buffer, embryos were dehydrated, embedded in plastic, and serially sectioned for transmission electron microscopy.

Criteria utilized to stage the embryos and descriptions of the embryos at various stages 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 at 27 °C since the beginning of the stage to the total number of days in the stage. A stage 9(3/4) embryo has completed the first 3 days of stage 9.

RESULTS

The leech is segmented and includes 21 essentially identical midbody segments. Each segment contains a ganglion which is linked to the periphery by a set of nerves; successive ganglia are linked by connectives (Muller, Nicholls & Stent 1981). The skin of the leech is innervated by a number of mechanosensory neurons whose cell bodies reside in the ganglia (Nicholls & Baylor, 1968; Kramer & Goldman, 1981). The primary peripheral axon of the dorsal pressure-sensitive neuron (P_D) mainly innervates half the dorsal skin of a segment ipsilateral to the P_D cell body via the dorsal posterior (DP) nerve in *Haementaria ghilianii* (Kramer & Kuwada, 1983).

The primary peripheral axon of the P_D is one of the earliest peripheral axons to develop (Kuwada & Kramer, 1983). Initially (stage 9(2/4)) the P_D neuron projects up to five peripherally directed growth cones which can radiate anteriorly, laterally, and posteriorly but rarely towards the ventral midline and they extend up to 50 μm from the cell body (Fig. 1A). However, approximately 1 day later a single primary peripheral axon, presumably developed from one of the initial neurites, is quite evident with its growth cone growing directly to the dorsal germinal plate which will later develop into the dorsal body wall and skin (Fig. 1B). Later the other initial peripheral neurites are eliminated (Kuwada & Kramer, 1983; Fig. 3A).

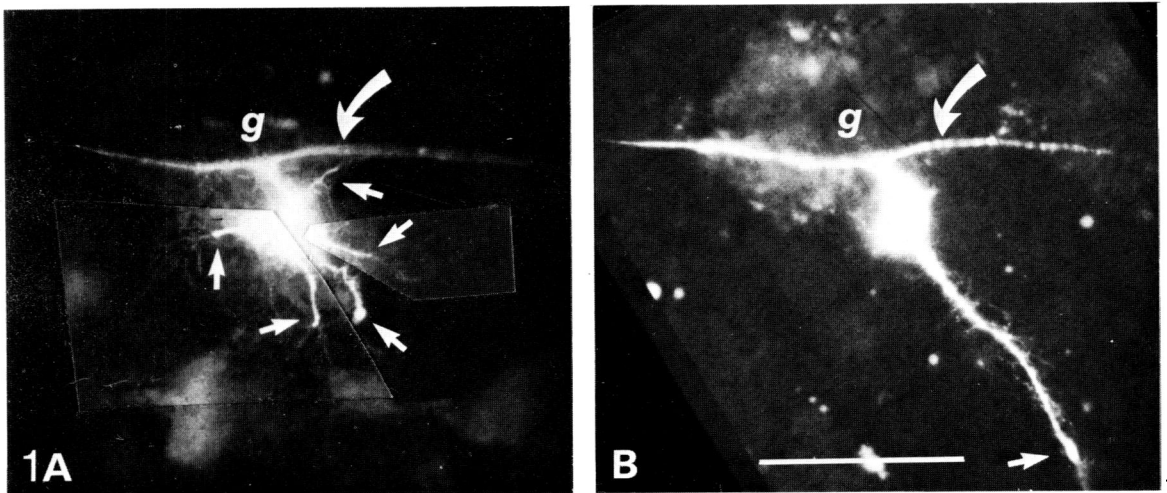


Fig. 1. The development of the primary peripheral axon of the P_D neuron. (A) The numerous peripheral growth cones of a Lucifer-Yellow-filled P_D neuron from a midbody ganglion in a stage 9(2/4) embryo. (B) The single peripheral growth cone of a midbody P_D neuron from a stage 9(3/4) embryo. *g* refers to the ganglion containing the P_D cell body. The curved arrow marks the central axon. Arrows point to the peripheral growth cones. All the growth cones are out of the ganglion; in some cases growth cones are on the germinal plate underneath the ganglion. The outline of the P_D cell bodies are not clear due to the halo from the brightly fluorescent cell bodies. Anterior is to the left. Scale 100 μm .

The pathway of the primary growth cone of all P_D neurons is stereotyped from segment to segment and from animal to animal. This, in conjunction with the early behaviour of the numerous initial peripheral growth cones, suggests that the P_D neuron establishes a primary peripheral axon by utilizing a specific substrate or guidance cue: one of the early growth cones finds an attractive substrate or cue which will lead it to the appropriate target area of the germinal plate (nascent body wall and skin) while the other growth cones are eliminated (Kuwada, 1982; Kuwada & Kramer, 1983). The pathway of the primary peripheral growth cone entails a number of cells and structures which may serve as an appropriate substrate and/or

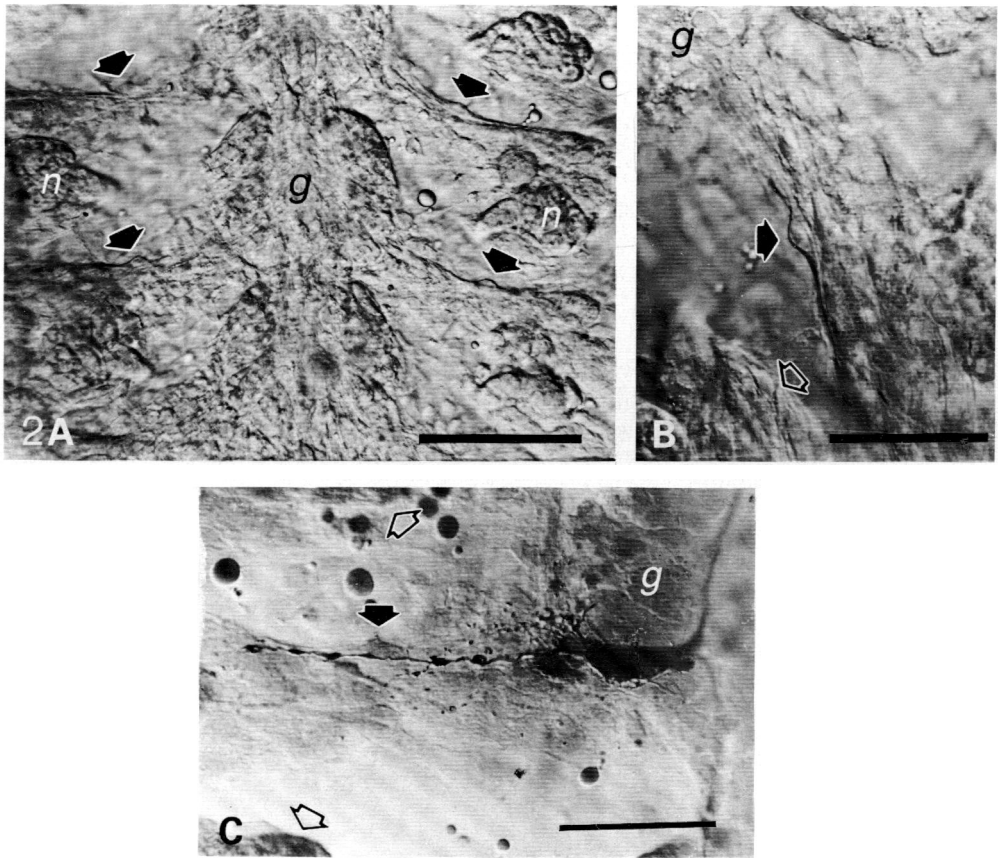


Fig. 2. The characteristic location of the DV cell. (A) A Nomarski optics photograph of several segments of a stage 9(3/4) embryo showing the relationship amongst the ganglia (*g*), nephridia (*n*), and the DV cell bodies (arrows). The nephridia are located just medial to the future lateral edge which separates the ventral and dorsal body wall and skin. Anterior is up. Scale 100 μ m. (B) A higher power view of a single DV soma denoted by the closed arrow. The open arrow indicates a nephridium and *g* a ganglion. Anterior is to the left. Scale 50 μ m. (C) A photograph from a stage 9(2.5-3/4) embryo demonstrating that the primary peripheral axon of the P_D neuron, which has been filled with HRP, runs right by the cell body of the DV cell (closed arrow). *g* denotes the ganglion and the open arrows the nephridia. Anterior is to the top. Scale 50 μ m.

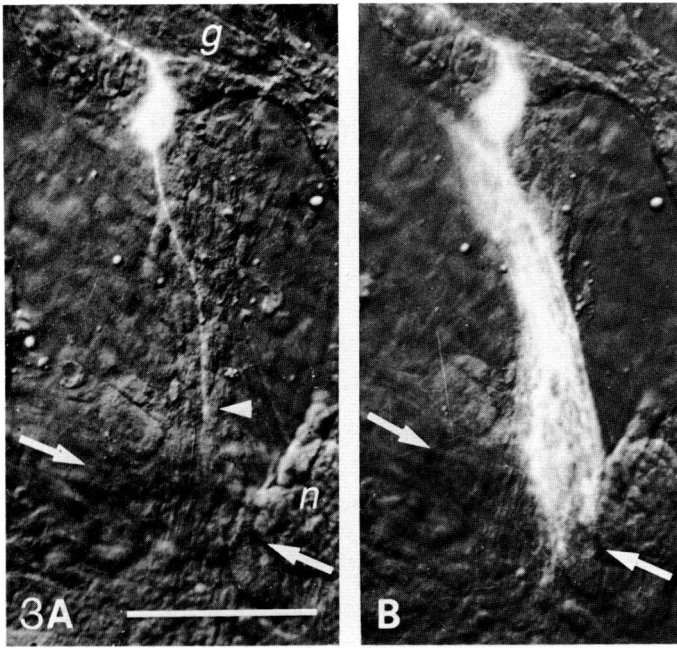


Fig. 3. The relationship between the peripheral axon of the P_D neuron and the DV cell. (A) A midbody P_D neuron filled with Lucifer Yellow from a stage 10(0/5) embryo viewed with combined fluorescence and Nomarski optics. The arrowhead denotes the P_D growth cone, arrows the future lateral edge, *n* a nephridium, and *g* a ganglion. (B) The same P_D neuron but now with the DV cell filled with Lucifer Yellow as well. Anterior is to the left. Scale $100\ \mu\text{m}$.

pathfinding cue (Kuwada, 1982). One of the most salient, the dorsoventral (DV) cell, has a relatively large, ovoid cell body ($5\ \mu\text{m}$ wide and $10\ \mu\text{m}$ long) located approximately half way between the ganglion and the future lateral edge (the boundary between the ventral and dorsal halves of the leech) (Fig. 3). The DV cell has a complex network of flat, thin processes approximately $30\ \mu\text{m}$ wide and $100\text{--}150\ \mu\text{m}$ long, which appear to insert into the ventral germinal plate near the ganglion, emerge onto the inner surface of the ventral germinal plate approximately $20\text{--}30\ \mu\text{m}$ from the ganglion, and insert into the dorsal germinal plate near the future lateral edge. Thus, the DV cell spans the territory which the primary peripheral growth cone of the P_D neuron must cross to get to its target area (Fig. 3B). Not only does the P_D axon run right by (Fig. 2C) and just under the DV cell body, but it can be seen to run along the entire length of the DV cell's flat processes (Fig. 3). The morphology of the DV cell is so unique that it is easily identifiable in sections viewed with the electron microscope (Fig. 4). In addition to its prominent cell body, it has a network of processes $50\ \text{nm}$ to $1\ \mu\text{m}$ in thickness which cover and demarcate a column of cells in the area where the flattener muscles will develop later. In the adult leech the dorsal

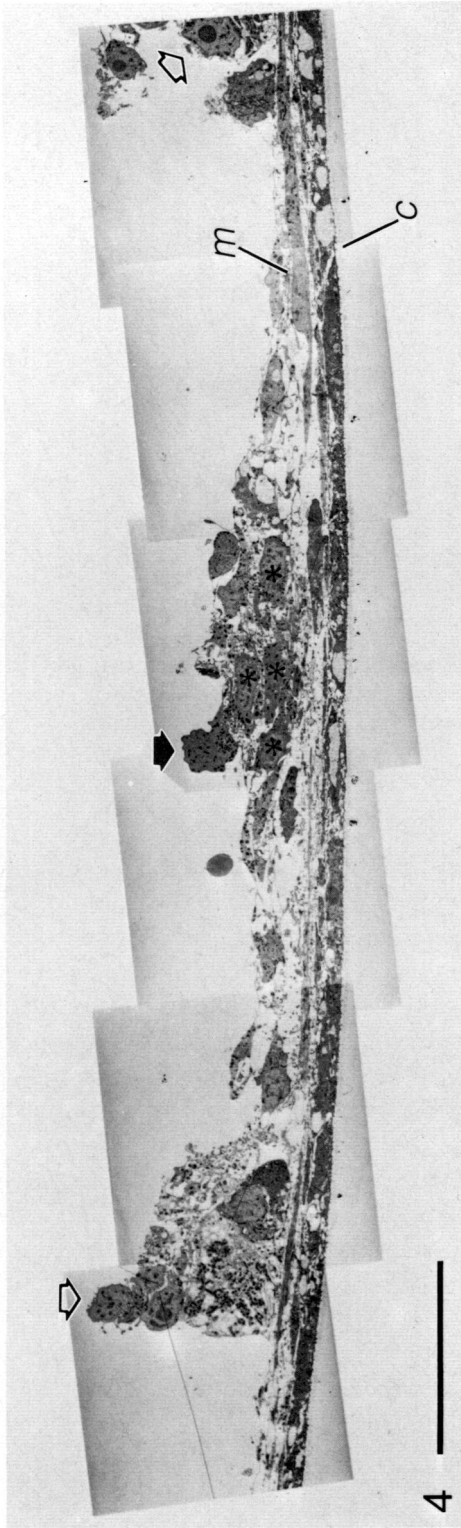


Fig. 4. An electron micrograph montage of a sagittal section of a midbody segment approximately $70\ \mu\text{m}$ lateral from the lateral edge of the ganglion in a stage 9(2.5-3/4) embryo showing the unique morphology and location of the DV cell body. This section was taken from the preparation shown in Fig. 2C at the level depicted by the closed arrow in Fig. 2C. Closed arrow denotes the cell body of the DV cell and the open arrows the nephridia from two adjacent segments. Asterisks mark some of the cells covered and demarcated by the processes of the DV cell (see text). *m* refers to a longitudinal muscle and *c* to the cuticle enveloping the embryo. Anterior is to the left. Scale $25\ \mu\text{m}$.

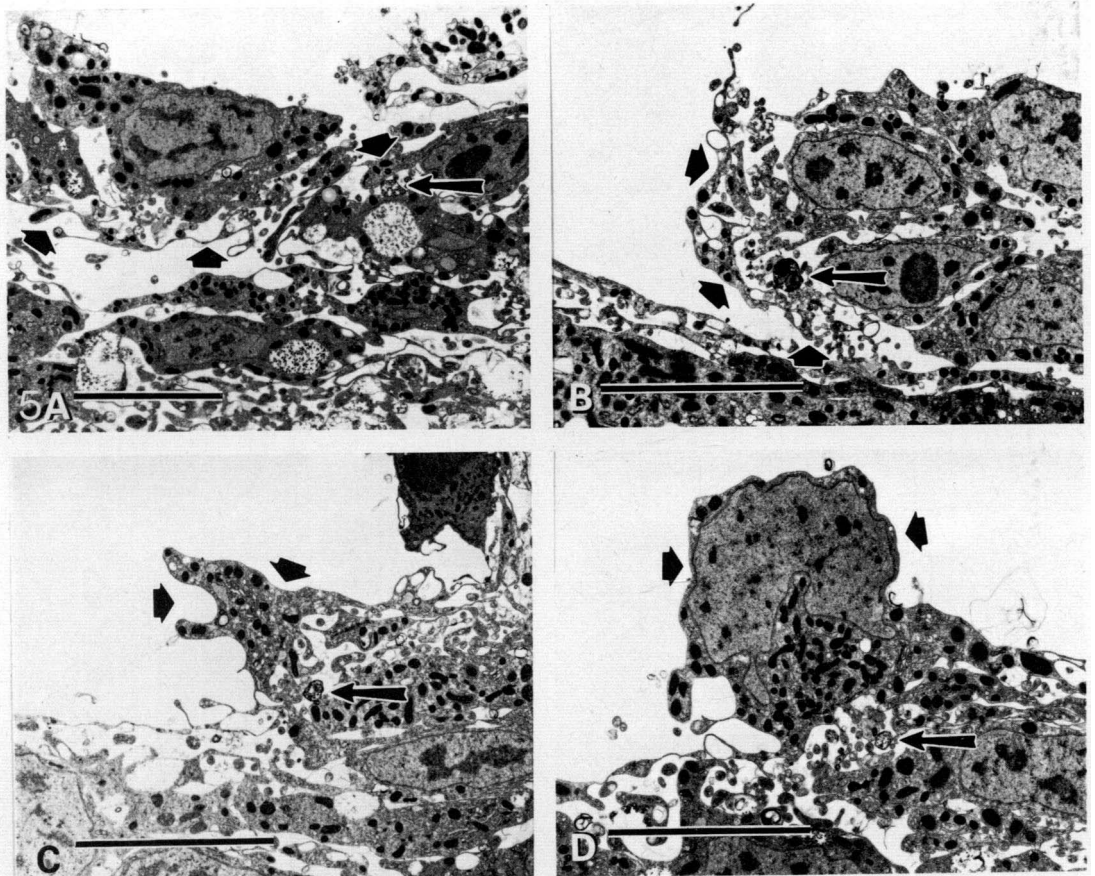


Fig. 5. Low power electron micrographs demonstrating the morphology of the DV cell and its association with an HRP-filled P_D axon in the stage 9(2.5–3/4) embryo shown in Fig. 2C. (A) At the level of the growth cone of the P_D neuron approximately 110 μm lateral from the edge of the ganglion. (B) Approximately 20 μm proximal from the growth cone. (C) Another 20 μm more proximal showing the lateral part of the DV cell body. (D) At the level of the nucleus of the DV cell. Short arrows denote the processes and soma of the DV cell and the long arrows the growth cone or axon of the P_D neuron. Anterior is to the left. Scale 5 μm .

posterior (DP) nerve, which contains the primary axon of the P_D neuron, is found embedded in the flattener muscles (Kuwada & Kramer, 1983). Additionally, the DV cell can be found in its normal position with its unique morphology at stage 9(1.5/4) when the P_D neuron has not yet projected any peripheral growth cones (Fig. 7B).

In three cases the P_D neuron from a midbody ganglion was filled with HRP in stage 9(2.5–3/4) embryos, when the primary peripheral axon is first evident, for examination with the electron microscope. In each case the growth cone of the P_D neuron and distal portions of the primary axon were more distal than any other growth cones or axons in the DP pathway. The P_D growth cone is, therefore, the

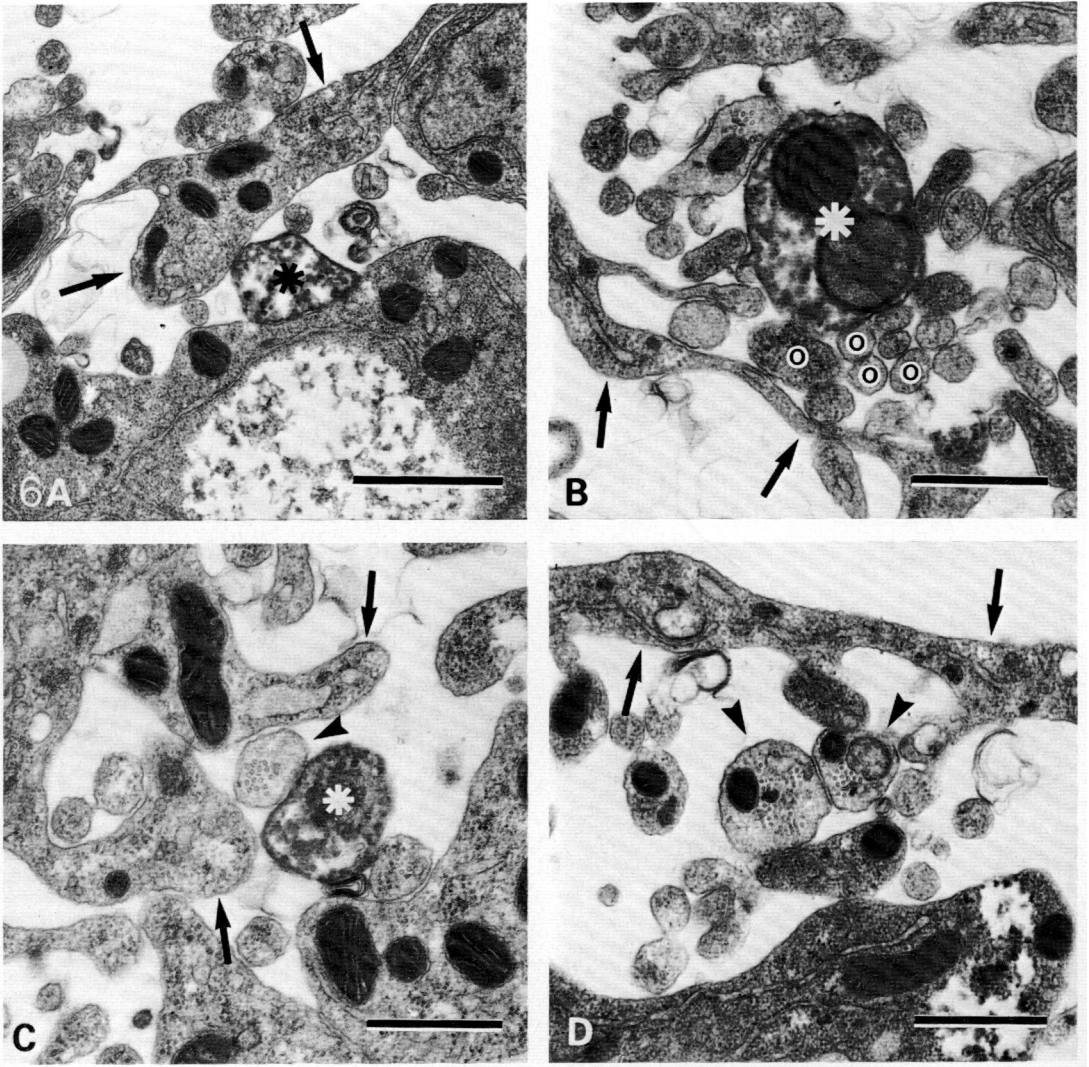


Fig. 6. Higher power electron micrographs showing that the P_D growth cone and axon contact the processes of the DV cell. (A) A higher power view of the growth cone (asterisk) from Fig. 5A. Arrows denote a process of the DV cell. Scale $1\ \mu\text{m}$. (B) A higher power view of the axon (asterisk) from Fig. 5B. The O's denote profiles of some of the processes of the DV cell. Scale $0.5\ \mu\text{m}$. (C) A higher power view of the axon (asterisk) from Fig. 5C. Arrowhead denotes a profile of another axon in the DP nerve. Scale $0.5\ \mu\text{m}$. (D) The DP nerve presumably containing the P_D axon from a proximal section of the next anterior segment showing two axon profiles (arrowheads) in contact with the processes of the DV cell (arrows) from that segment. Scale $0.5\ \mu\text{m}$. In all the micrographs anterior is to the left.

earliest growth cone to traverse this pathway (Fig. 5A,6A). Other DP axons fasciculated with the P_D axon more proximally (Fig. 6C). Axons were easy to distinguish from other cellular profiles, some of which belonged to the DV cell,

because of the regular array of microtubules which characterized the axons. In those same sections, the HRP filled P_D growth cone and axon were associated and in contact with the processes and cell body of the DV cell (Fig. 5,6). The DV cell processes were identified by following them from the DV cell body in serially sectioned preparations ($n=2$).

The DV cell has a resting potential of approximately -35 mV, input resistance of 2 megohms, and linear membrane characteristics (Fig. 8). The lack of electrogenic properties in addition to its unusual morphology suggests that the DV cell is a non-neuronal cell. Furthermore, it is dye coupled (Lucifer Yellow) to many of the cells found along the DP pathway which are covered by the DV processes (Fig. 7A), but was never found to be dye coupled to the P_D neuron nor any other neuron.

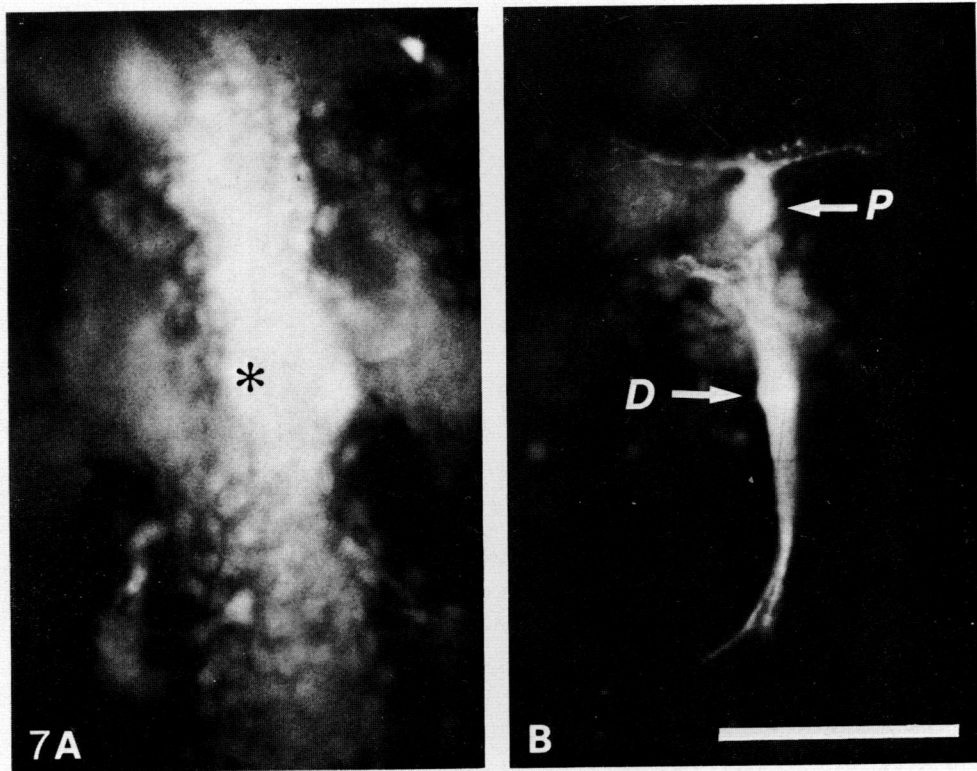


Fig. 7. (A) Dye coupling between the DV cell and smaller cells underneath and demarcated by the processes of the DV cell in a stage 10(0/5) embryo. Lucifer Yellow was injected into the DV cell body. The asterisk signifies the DV soma. The ganglion and nephridium are, respectively, at the top and bottom of the fluorescence photograph. (B) The DV cell is present with its unique morphology before the P_D neuron has projected any peripheral growth cones. Both the P_D neuron (P) and the DV cell (D) were filled with Lucifer Yellow in this 9(1.5/4) embryo. The P_D neuron at this stage has central processes but no peripheral ones. Anterior is to the left. Scale 100 μ m.

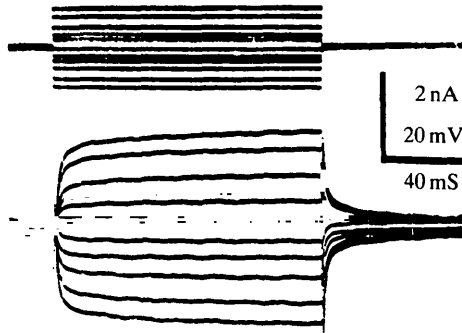


Fig. 8. Physiology of the DV cell from a stage 9(3/4) embryo. Top traces are records of the intracellularly injected current and the bottom traces the voltage responses. Eleven traces were superimposed. This cell had a resting potential of -35 mV.

DISCUSSION

Pathfinding by the primary peripheral growth cone of the P_D neuron.

Analyses of axonal growth by identifiable embryonic neurons have established that neurons make specific connections with each other by utilizing various external cues (Raper *et al.* 1983*a,b,c*; Ho *et al.* 1983; Bentley & Caudy, 1983; Berlot & Goodman, 1984; Raper *et al.* 1984; Bastiani *et al.* 1984). Likewise the manner in which the primary peripheral axon of the P_D neuron is established suggests that axonal guidance cues may be involved: an initial projection of an excessive number of peripheral growth cones followed by the rapid growth of one of the growth cones directly to its target area and the elimination of the others (Kuwada, 1982; Kuwada & Kramer, 1983). Consistent with this hypothesis are the two main findings of this investigation. 1) The primary growth cone of the P_D neuron is the first growth cone to traverse the DP pathway and, therefore, establishes the DP nerve. 2) Its growth cone and axon grow in contact with the processes of the large, non-neuronal DV cell. Furthermore, the DV cell possesses a unique morphology which makes it especially suitable as a substrate and/or guidance cue and is present with its unique morphology in its characteristic position before the P_D neuron has projected any peripheral growth cones. These characteristics suggest that the DV cell may be involved in the guidance of the pioneer P_D growth cone.

In the embryonic grasshopper nervous system neurons project the correct number of growth cones and they seem to make pathfinding decisions on the basis of selective filopodial contact (Raper *et al.* 1983*c*; Taghert *et al.* 1982; Bastiani & Goodman, 1984; Bastiani *et al.* 1984). Many neurons in the embryonic leech also project the correct number of growth cones which proceed to grow in a stereotyped and regular fashion (Kuwada & Kramer, 1983; Kramer & Kuwada, 1983; Kuwada, 1984). The establishment of the primary peripheral axon of the P_D neuron is

unusual because an excessive number of growth cones are initially projected. Furthermore, the other peripheral axons of the P_D neuron, which are projected later, and the central axon of the P_D neuron, which is projected before any peripheral growth cones, are all established by extension of a single growth cone (Kuwada & Kramer, 1983; Kramer & Kuwada, 1983; Kuwada, 1984). The reason for the difference between the manner in which the P_D primary peripheral axon and many other embryonic axons are established is not known. One possibility is that the guidance cue is beyond reasonable filopodial reach. Therefore, the projection of multiple growth cones may be necessary in order for filopodial contact with the appropriate cue. This point of view appears to conflict with the notion that the DV cell is the guidance cue since its processes are found in the vicinity of the ganglion prior to extension of peripheral growth cones by the P_D neuron. However, these processes appear to be inserted into the germinal plate or developing body wall near the ganglion and emerge onto the inner surface of the nascent body wall approximately 20–30 μm lateral to the ganglion. Thus, the closest DV processes may not be readily accessible to the filopodia of the P_D neuron. The DV processes more lateral are accessible but may be far enough from the ganglion to require projection of multiple growth cones in order to insure filopodial contact.

Is the DV cell a muscle pioneer?

The embryonic grasshopper contains large mesodermal cells known as muscle pioneers with morphologies similar to the DV cell (Ho *et al.* 1983). Muscle pioneers apparently function as a scaffold for organizing specific muscles and as axonal guidance cues for outgrowing motor neurons which normally innervate those muscles. It is possible that the DV cell may function as a muscle pioneer in the embryonic leech for a number of reasons. (1) The DV cell is probably of mesodermal origin since the Lan 3–14 monoclonal antibody, which recognizes leech muscles (Zipser & McKay, 1981; Stuart, Thompson, Weisblat & Kramer, 1982), recognizes the DV cell (unpublished results). (2) It occupies the location where the dorso-ventral flattener muscles will develop and covers and demarcates a column of cells some of which may develop into the flattener muscles. (3) It may serve as an axonal guidance cue for the pioneering P_D growth cone and the DP nerve. Growth cone guidance by mesodermal derivatives both in the CNS and periphery has been previously suggested in the embryonic leech (Kuwada, 1982; Kuwada & Kramer, 1983; Kramer & Kuwada, 1983). This suggestion awaits experimental confirmation, and the large and conspicuous nature of the DV cell makes it attractive for future manipulations.

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