

# PATHFINDING BY NEURONAL GROWTH CONES IN GRASSHOPPER EMBRYOS

## IV. The Effects of Ablating the A and P Axons Upon the Behavior of the G Growth Cone<sup>1</sup>

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### Abstract

In the companion paper (Bastiani, M. J., J. A. Raper, and C. S. Goodman (1984) *J. Neurosci.* 4: 2311-2328), we show that as the G growth cone reaches its choice point and turns anteriorly on the A/P fascicle, its filopodia demonstrate selective affinity for the A/P fascicle as compared to the other approximately 25 longitudinal axon fascicles, and within the A/P fascicle itself, G's filopodia selectively contact the P axons as compared to the A axons. These results support the hypothesis that the A/P fascicle, and, moreover, subsets of axons within it (Ps versus As), are specifically labeled and that the G growth cone is determined to follow a particular labeled pathway.

We tested the "labeled pathways" hypothesis by specifically ablating these axons and examining the subsequent behavior of the G growth cone in embryos grown in culture. Ablation of the A and P axons prevents G from growing more than a short distance anteriorly, although the G growth cone is within grasp of many other longitudinal axon fascicles. Ablation of only the P axons has a similar effect; the G growth cone behaves normally if only the A axons are ablated.

Transmission electron micrograph semiserial section reconstructions of experimental embryos further indicate that G's growth cone behaves abnormally when the A and P axons, or only the P axons, are ablated. The G growth cone branches extensively in the lateral and ventral neuropil without it or its filopodia showing a high affinity for any other axon fascicle. These results indicate that the G growth cone is able to distinguish the A/P fascicle from the other longitudinal axon fascicles in the developing neuropil. Moreover, the results suggest that within the A/P fascicle the G growth cone is able to distinguish the P axons from the A axons.

We previously described the morphogenesis of the G neuron in the central nervous system of the grasshopper embryo (Raper et al., 1983a). As the G growth cone turns anteriorly, its growth cone always fasciculates with a discrete bundle of axons in preference to other nearby bundles. We were also able to identify the A1, A2, P1, and P2 neurons which establish this axon bundle, called the A/P fascicle (Raper et al., 1983b). The axons of the A1 and A2 cells extend anteriorly through the dorsal lateral neuropil. They meet and fasciculate with the two posterior growing axons of the P1 and P2 neurons. In this paper we describe a third neuron, the P3 cell, whose axon joins the fascicle just after P1 and P2.

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In the companion paper we show that as G reaches its choice point and subsequently extends anteriorly upon the A/P fascicle, its filopodia are more often in contact with and appear to prefer the A/P fascicle over the other longitudinal axon fascicles (Bastiani et al., 1984). Within the A/P fascicle itself, the tip of G's growth cone is found to be closely associated with the P and not the A axons. Furthermore, before the G growth cone climbs onto the A/P fascicle, its filopodia show a significant preference for the P axons as compared to the A axons. These findings suggest that G is able to distinguish the A/P fascicle from other axon bundles and, moreover, is able to distinguish the P axons from the A axons.

In this study we examine the effects of ablating the A1, A2, P1, P2, and P3 axons upon the morphogenesis of the G neuron. If the A/P fascicle guides G's growth cone anteriorly through a specific location in the neuropil, then its ablation should prevent G's normal anterior extension. If G's growth cone is determined to elongate upon particular axons within the fascicle, then only the ablation of those particular axons should affect G's behavior. Our results suggest that the A/P fascicle plays an important role in guiding G anteriorly through the neuropil and that it is the P axons specifically which appear to be most active in this role.

### Materials and Methods

Acute dissections, visualization of embryos, and intracellular injections of the fluorescent marker Lucifer Yellow have been described previously (Raper et al., 1983a). Lucifer Yellow injections were observed directly in living embryos by fluorescence and drawn immediately with the aid of a Zeiss drawing tube. A rabbit serum antibody to Lucifer Yellow (anti-LY) was used in processing preparations for photography (Taghert et al., 1982). The anti-LY antibody was visualized with the anti-rabbit biotin/avidin/HRP reagents supplied in Vectastain kit PK-4001. The intracellular injection of horseradish peroxidase (HRP) and the processing of tissue for transmission electron microscopy (TEM) have also been described (Raper et al., 1983b; Bastiani et al., 1984).

The base embryo culture medium used for these experiments consisted of RPMI 1640 (Gibco) supplemented with 2 gm/liter of sodium bicarbonate, 0.29 gm/liter of glutamine, 0.11 gm/liter of sodium pyruvate, 2.0 gm/liter of glucose, 50,000 units of penicillin, and 50,000 mcg of streptomycin. This medium was then diluted with 10% horse serum and further supplemented with 3.0 gm/liter of glycine, 7.8 mg/liter of bovine insulin (Sigma), 150  $\mu$ g/liter of  $\beta$ -ecdysterone (Sigma), and 0.5  $\mu$ g/liter of juvenile hormone I (Sigma). Cultures were maintained in a moist incubator in a 5% CO<sub>2</sub> atmosphere at 29°C.

Embryos were cultured in an open sterile hood by the following procedure. First, an individual egg was sterilized by immersion into a concentrated solution of sodium hypochlorite (Chlorox). As soon as the chorion cleared, the egg was washed once in fresh medium and then moved into a Petri dish containing approximately 10 ml of culture medium. From this point onward, sterile dissection tools were used throughout.

The embryo was removed from the egg and mounted upon a glass Sylgard coffin slide just as in acute experiments. The slide was then placed upon the stage of a compound microscope, and the embryo was examined with a Leitz  $\times$  50 water immersion lens and Nomarski optics. No special precautions were necessary to keep the preparation sterile other than briefly swabbing the microscope stage and lens in 70% ethanol.

The ablation of neuronal cell bodies and axons was accomplished with a sharp microelectrode. The axons of the A1 and A2 cell bodies in the third thoracic ganglion were prevented from advancing into the neuropil of the second thoracic ganglion by cutting the lateral portion of the connective joining the two ganglia (Fig. 1). First, either of the A1 or A2 neurons were filled with Lucifer Yellow. This marks both neurons since the pair is dye coupled. The preparation was then viewed under fluorescence very briefly (about 1 sec) to verify that the proper neurons had been filled. Then the lateral connective was cut, and the preparation was viewed once more to verify that both the A1 and A2 axons were severed. The P1, P2, and P3 cell bodies in the second thoracic ganglion were killed in a similar manner. First the P2 neuron was filled with Lucifer Yellow. The preparation was then observed under fluorescent illumination with the field stop diaphragm confining illumination to only the region immediately surrounding the P neurons. Since the P1 and P3 neurons are dye-coupled to the P2 at this age, all three cells fluoresced after this one fill. The P1, P2, and P3 cell bodies were each in turn punctured with a microelectrode, and their cell bodies were separated from their axons by rapidly advancing the electrode anteriorly (Fig. 1). This procedure took from 15 to 30 sec. All manipulations were confined to one side of the nervous system so that the other side served as an internal control. If at any point an incorrect cell was filled with Lucifer Yellow or a kill was not accomplished with certainty, the preparation was discarded.

The manipulated embryo was then transferred to a Petri dish containing fresh medium and the sterile filter assembly upon which it would ultimately be cultured. The filter assembly consisted of a small rectangular block of Teflon 18 mm long, 6 mm wide, and 3 mm high. A hole 3 mm in diameter was drilled from top to bottom through the block. A portion of the block directly underneath this hole was milled away. When viewed from the side and slightly above, the entire assembly has the appearance of a bridge with a hole in its center. The top of the hole was covered with a 0.1- $\mu$ m polycarbonate filter (Nucleopore) glued to the Teflon surface with RTV silicone rubber cement (General Electric). While the filter assembly was entirely submerged, the dissected embryo was placed with its dorsal side against the filter. The entire assembly was then grasped in a pair of forceps and pulled through the air-water interface, flattening the embryo down against the filter. The assembly was then placed in a Petri dish (Falcon 1008) with sufficient medium (2.7 ml) so that the embryo rested at the air-water

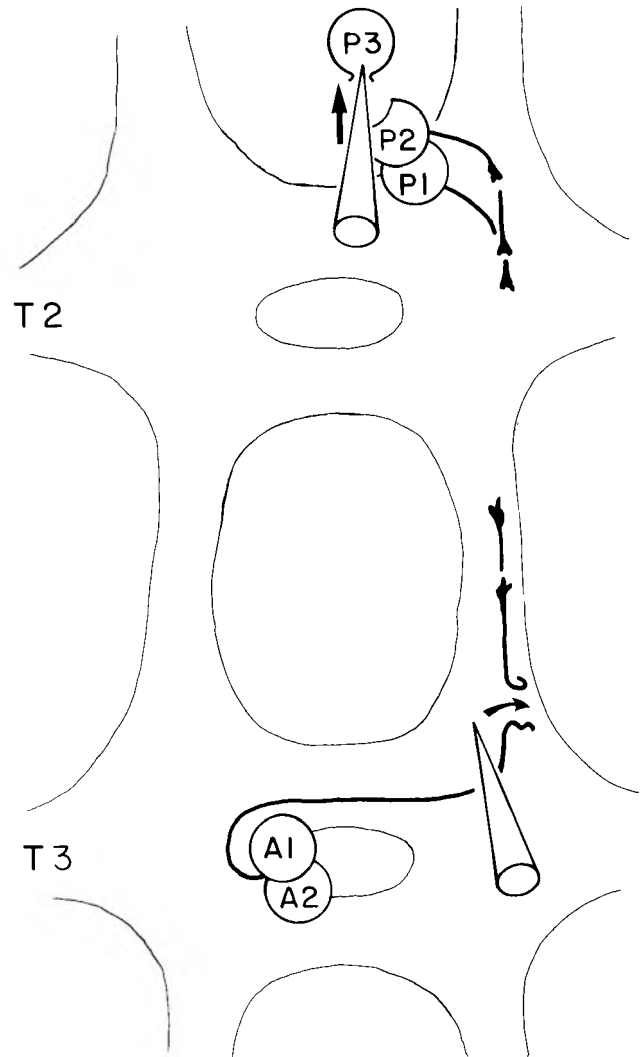


Figure 1. Schematic diagram of the procedure employed to sever the axons of the A1 and A2 neurons and to kill the P1, P2, and P3 neurons (see the text for details).

interface. Two conditioning embryos (see below) were added to the culture media and were replaced daily for as long as the manipulated embryo was cultured.

Cultured embryos were removed from the filter assembly by floating them onto the surface of saline (composition given by Raper et al., 1983a). All subsequent procedures were then as for acute preparations.

Conditioning embryos were made in the following manner. One day before experimental manipulations were performed, 55% embryos were sterilized and washed as described. The embryos and approximately one-half of their associated yolk were removed from the egg shell by the method of Mueller (1963). These embryos were cultured overnight in medium containing no juvenile hormone or  $\beta$ -ecdysterone. After 1 day in culture the serosal membrane healed over all of the remaining yolk, and the embryo was ready for use. Conditioning embryos could sometimes be kept for as long as a week before they were used, as long as they were not exposed to juvenile hormone and  $\beta$ -ecdysterone.

### Results

*Identification of the G, A, and P neurons.* In order to carry out these experiments, it was imperative that we be able to locate the cell bodies of the G, A, and P neurons in nearly every preparation. The cell body of the G neuron can be identified as described previously (Raper et al., 1983a). We have further characterized the positions of the A1, A2, P1, and P2 cells, and in the process we have discovered one of the next neurons that

contributes an axon to the A/P fascicle. The A1 and A2 cell bodies are found in the third thoracic ganglion on the side contralateral to their axons (Raper et al., 1983b). They generally lie among the most dorsal cell bodies underneath the developing neuropil. They are found directly below the vMP2 axon fascicle on each side of the embryo, just anterior to the dMP2 neuron, and are amongst the largest and clearest neuronal cell bodies in the vicinity (if the easily identified MP1-3 neurons are discounted) at the ages employed in this study. After filling the A1 and A2 neurons with Lucifer Yellow, their axonal morphologies are distinctive and can be easily distinguished from those of their neighbors by the fascicles in which their axons run.

The P2 cell body is found in the second thoracic ganglion on the side ipsilateral to its axon. It is just medial to the portion of the neuropil where the most anterior commissure and the ganglionic connectives meet (Fig. 2). P2 is particularly easy to locate because it is large, clear, and lies directly beneath the neuropil. An axon fascicle rises from deeper cell bodies and passes just posterior to the P2 cell body before it loops into the anterior commissure. The P1 cell body lies just ventral and posterior to the P2 cell body. It is the smaller and the most posterior of a pair of elongated neurons. A third neuron which contributes an axon to the A/P fascicle, the P3 cell, lies just medial and dorsal to the P2 cell.

The P1 neuron is the first of the P cells to extend an axon posteriorly (Fig. 2A). It takes a relatively ventral route in the neuropil until it rises dorsally toward the basement membrane near the lateral intrasegmental nerve root. The P2 and P3 neurons extend axons laterally to the most dorsal portion of the neuropil. Their axons then turn posteriorly, fasciculating with the axon of the P1 neuron (Fig. 2B). In our previous description of these events at the light level (Raper et al., 1983b) we probably confused the identities of the P1 and P2 neurons, describing them as if they were one cell. We thereby underestimated the time of P1's arrival to the point at which G turns anteriorly. Therefore, it is important to note that the P1 axon is moving through G's choice point at about the same time that G turns anteriorly (Fig. 2C).

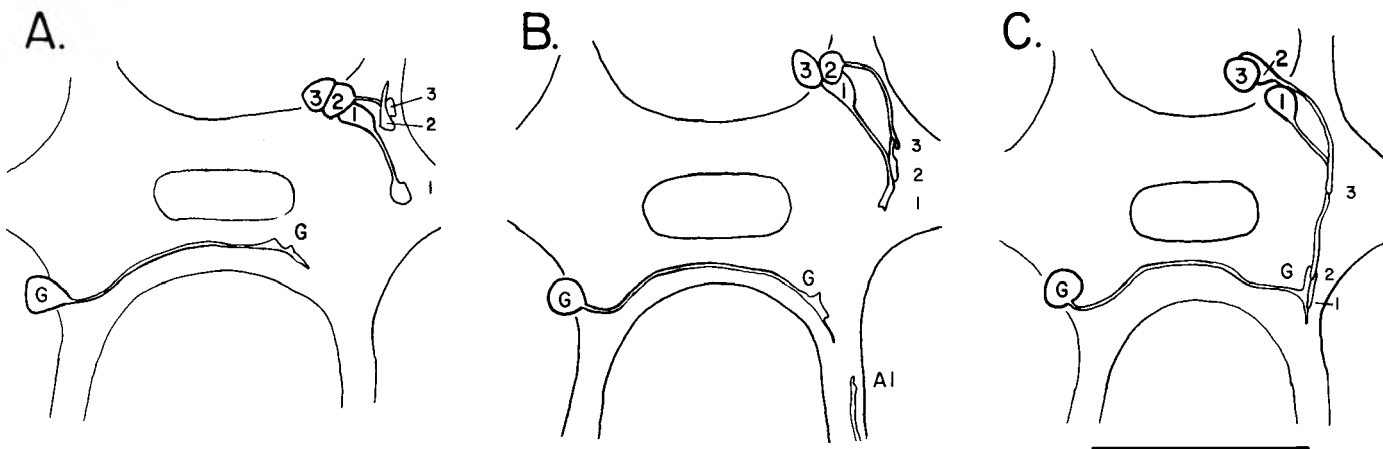
**Development of the G neuron in culture.** The experimental manipulations described in this study were performed during a relatively narrow time window (*i*) after the growth cones of both the A1 and A2 neurons had turned anteriorly into the nerve connective that joins the third and second thoracic segments, and (*ii*) before the growth cones of the G, A1, or P1

neurons reached the location at which G turns anteriorly. The relative positions of the G, A, and P growth cones from a representative preparation are shown in Figure 3A. At this stage of development (approximately 37%) the neuropil contains a limited number of axon bundles (see Fig. 3 in Bastiani et al., 1984). If an embryo is removed from its egg and cultured at 29°C for 40 to 48 hr, G's growth cone continues extending to a lateral position in the neuropil and then turns onto the A/P fascicle and extends anteriorly (Fig. 3B). In favorable cultures, the distalmost tip of G's growth cone advances anteriorly into the ganglionic connective joining the second and first thoracic segments (equivalent to 42% of embryogenesis). Thus, we are able to attain sufficient development in culture to examine the cues which guide G's growth cone through its choice point and anteriorly through the neuropil of the second thoracic ganglion.

Although G turns anteriorly in the A/P fascicle, G's morphogenesis in culture is not entirely normal. Occasionally, in culture, the G axon has one or several short branches extending laterally from the main axon further anterior from the choice point. For example, the two lateral branches shown in Figure 3B are usually not seen *in ovo*. Another more common error in G's development is found in culture. G's primary growth cone turns in an almost exclusively anterior direction *in ovo* (Raper et al., 1983a). However, in culture, G's growth cone often branches and extends both a long neurite anteriorly and a shorter neurite posteriorly (e.g., Fig. 4A, discussed further with reference to Fig. 6). One final difference sometimes observed between cultured and *in ovo* embryos involves the tightness with which axons fasciculate into bundles. Many new axons are added to the neuropil while in culture (e.g., Fig. 10A). Axons are organized into their appropriate bundles, and these fascicles are in the appropriate locations. In some cases, the axon fascicles are as tight as *in ovo* (e.g., Figs. 10B and 11B). However, in other cases, the axon fascicles are not as tight or subdivide into smaller tight bundles that loosely associate with one another (e.g., Fig. 15).

#### Light level observations of experimental embryos

**Effects of ablating the A and P axons.** The axons of the A1, A2, P1, P2, and P3 neurons were prevented from making the A/P fascicle in the second thoracic ganglion by (*i*) cutting the portion of the ganglionic connectives in which the A1 and A2 axons run and (*ii*) by killing the P1, P2, and P3 cell bodies and separating them from their axons (see "Materials and Meth-



**Figure 2.** Normal development of the P1, P2, and P3 neurons in the second thoracic ganglion. *A*, Approximately 36% of development *in ovo* the P1 cell extends its axon posteriorly by a relatively ventral route, while the P2 and P3 neurons extend axons only laterally by a dorsal route. *B*, By approximately 37% the P2 and P3 axons have grown posteriorly and begun to fasciculate upon the P1 axon. The growth cone of the A1 cell is advancing rapidly toward the point at which G turns anteriorly. *C*, At approximately 40% G has just begun to extend anteriorly in the fascicle formed by the A1, A2 (not shown), P1, P2, and P3 axons. Calibration bar: 100  $\mu$ m.

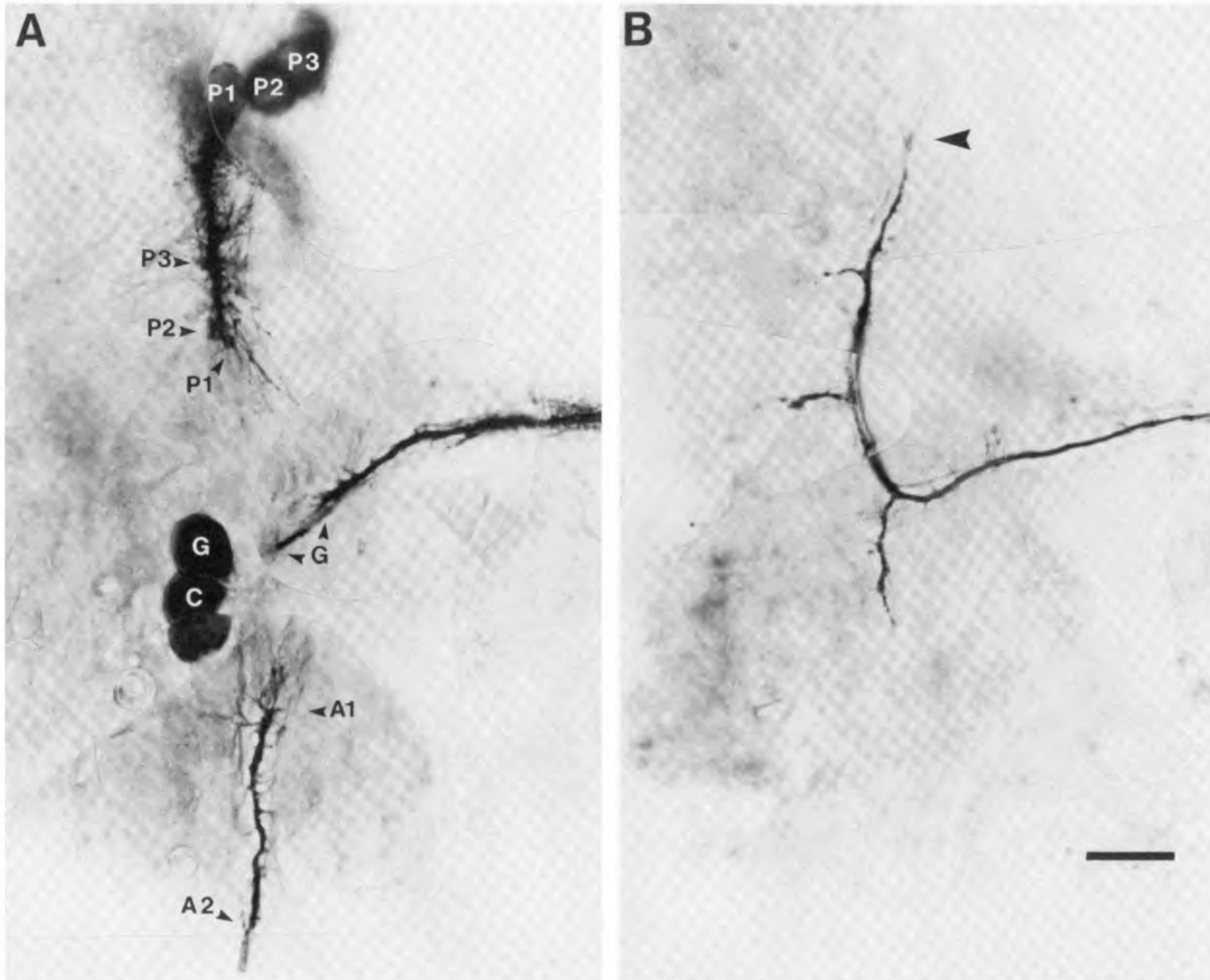
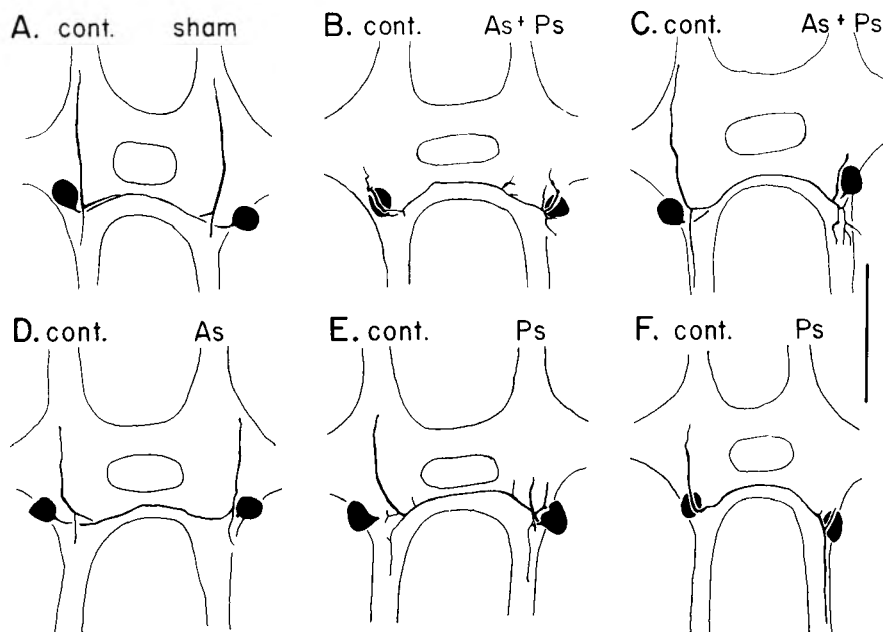


Figure 3. *A*, The relative positions of the A1, A2, P1, P2, P3, and G growth cones at the time when experimental manipulations were made. Positions of the growth cones are indicated and correspond to those in Figure 2*B*. The midline of the ganglion is to the right, and anterior is to the top. The cell bodies of the G, C, and Q5 neurons were dye-coupled to the filled G cell (out of the frame to the right). *B*, The morphology of the G cell in a control embryo cultured for 2 days at 29°C. Both photographs are to the same scale. Calibration bar: 25 μm.



**Figure 4.** The morphology of the G neuron in examples of experimental embryos. The embryos were cultured for 40 to 48 hr after cellular ablations. All manipulations were performed on the right sides, leaving the left sides as internal controls. Each panel shows the right cell body and its left contralateral control axon, and the left cell body and its right contralateral experimental axon. Note only one axon is drawn at the midline because both axons tightly fasciculate. *A*, Sham-manipulated. *B*, A1 and A2 axons cut; P1, P2, and P3 neurons ablated. In this embryo the control G is relatively undeveloped. *C*, A1 and A2 axons cut; P1, P2, and P3 neurons ablated. In this embryo the control G is relatively developed, and the experimental G develops abnormally. *D*, A1 and A2 axons cut; the experimental G behaves normally. *E* and *F*, P1, P2, and P3 neurons ablated; the experimental G behaves abnormally. Calibration bar: 100  $\mu$ m.

ods"). Manipulations were performed on one side of the embryo only, so that the opposite side served as an internal control. The embryos were cultured at 29°C for 40 to 48 hr. Upon removal from culture, the G neurons on both sides of the second thoracic ganglion were filled with Lucifer Yellow. In sham controls, the same manipulations were performed in exactly the same manner except that (i) the medial portion of the ganglionic connective was severed instead of the lateral portion, and (ii) cells just medial and dorsal to the P1, P2, and P3 cell bodies were killed (most of these adjacent neurons are motoneurons whose axons do not extend anywhere near the Ps).

It should be noted that our experimental procedure was more invasive than we would have liked. It is likely that a few other axons were cut in the lateral connective in addition to those of the A1 and A2 cells. It is also likely that a few other cells may have been damaged or killed in the neighborhood of the P1, P2, and P3 cell bodies. We tried to minimize any damage to the neuropil where G normally joins the A/P fascicle by inserting the microelectrode used to fill and kill the P neurons far away from this neuropil region.

The morphologies of a control G and sham-manipulated G are shown in two-dimensional camera lucida drawings in Figure 4*A*. The axons of both neurons extend well anteriorly through the normal, lateral portion of the neuropil. In Figure 4*B* is shown a preparation in which the A1, A2, P1, P2, and P3 axons were ablated on one side. This is a preparation in which the control G appears relatively immature. Both the control and manipulated G cells have extended a short distance anteriorly. Numerous primary growth cones and their axons are seen on the experimental as compared to the control side. In Figure 4*C* is shown another preparation in which the A1, A2, P1, P2, and P3 axons were ablated on one side. In this preparation the control G extends anteriorly past the anterior commissure,

while the experimental G has multiple growth cones and extends anteriorly for only a short distance. Both Gs have significant posteriorly directed branches. The multiple posteriorly directed growth cones of the experimental G appear to wander and branch anomalously. For another example of the effects of ablating the A and P cells upon G's morphology, see Figure 7.

*Effects of ablating subsets of axons in the A/P fascicle.* No effect upon G's behavior was detected if only the A1 and A2 axons were prevented from joining the A/P fascicle (Fig. 4*D*). By injecting the A cells with Lucifer Yellow prior to cutting their axons, we were able to look later with fluorescence and determine that the distal growth cone and axon degenerates into small vesicles and, moreover, that fusion of the proximal and distal stumps has never been observed. Furthermore, it is unlikely that the A1 and A2 cells replaced the cut axons. In pilot experiments in which unfilled A1 and A2 axons were cut with a pumped-dye laser (Phase-R) focused to a 1- $\mu$ m beam through a microscope (Taghert et al., 1984), the A cells were found to have not resprouted in five preparations, to have resprouted and joined the wrong fascicle in the wrong connective in one preparation, and to have axons advancing normally in the correct portion of the correct connective in four preparations. There was no evidence of damage to the A axons in these last four preparations (as there was in all other preparations examined); therefore, we think it likely that they were missed by the laser altogether. In summary, ablating the A1 and A2 axons had no effect upon the behavior of the G growth cone.

Ablation of only the P cells does effect G's behavior. In Figure 4*E* is shown a preparation in which the P1, P2, and P3 cells were killed on the right side. The control G has made considerable progress anteriorly, while several growth cones on the experimental side make considerably less anterior progress.

The two more medial of these processes are clearly growing in abnormally medial positions. In another preparation the G cell extends almost exclusively in a posterior direction on the experimental side of the neuropil (Fig. 4F). For two additional examples of the effects of ablating the P cells on G's morphology, see Figures 9 and 14.

*Comparing the effects of axon ablations.* One source of variability from preparation to preparation comes from the variable stretching of embryos as they are flattened onto the polycarbonate filter during culture or as they are pinned out before they are assayed. Thus, the absolute lengths of the G neurites were normalized to the measured distance between the anterior margin of the anteriormost commissure and the posterior margin of the posterior commissure at the embryonic midline.

The effects of differing manipulations are more easily compared if the anterior progress of the experimental G is plotted as a function of the anterior progress of the control G for each experimental condition. This plot was derived from the following considerations. The experimental manipulations were performed on embryos of similar, but not identical, ages. Those placed in culture at a relatively earlier age would not be expected to develop to as late a stage as those placed into culture at a later age. It was also evident as the experiments were performed that some embryos, or batches of embryos, responded better to the culture conditions employed than others. Both of these sources of variability in the data are effectively normalized by plotting the experimental length as a function of the control length, since both should be affected equally by

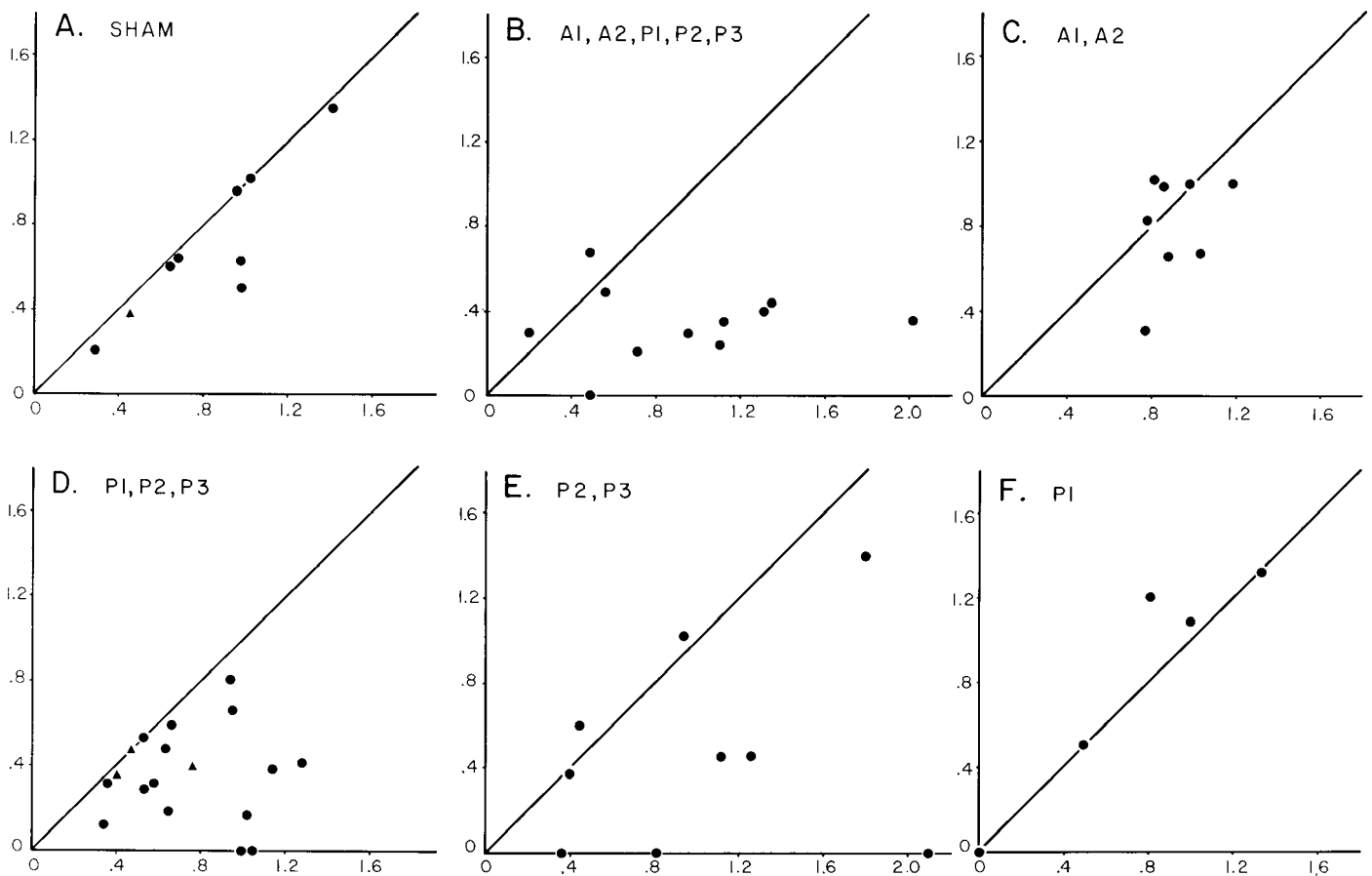
these two factors. When plotted in this manner, manipulations which have no effect should produce points which cluster around a straight line running through the origin and with a slope of 1.

The relative anterior progress of G cells on the control sides as compared to the sham-manipulated sides is very nearly the same for all preparations (Fig. 5A). This indicates that the relatively invasive experimental procedures employed in this study cannot account for the effects of ablating the A and P neurons.

Ablation of the A1 and A2 axons, together with the P1, P2, and P3 neurons, clearly has a significant effect on G's ability to extend anteriorly. The more developed the control side, the more obvious is the deficit on the experimental side (Fig. 5B). This suggests that the G easily traverses the choice point at which it turns anteriorly without the aid of the A or P axons, but that it rarely proceeds more than a short distance anteriorly in their absence.

Cutting only the A axons appears to have no significant effect upon G's anterior progress (Fig. 5C). Killing only the P1, P2, and P3 neurons produces fairly variable results, which, as a whole, indicate that the absence of the P cells does effect G's anterior progress (Fig. 5D). We were able to verify in three preparations that at least one A axon had traversed the second thoracic neuropil.

In an effort to determine whether some subset of the P cells is primarily responsible for this effect, we ablated the P1 cell alone or the P2 and P3 cells alone. These manipulations are



*Figure 5.* Anterior extension of the G axons on the experimental (Y axis) as compared to the control (X axis) sides of manipulated and cultured embryos. Lengths are expressed in arbitrary dimensionless units (see the text). A, Sham-manipulations. The circles indicate medial portion of ganglionic connective cut and cells adjacent to the P neurons killed. The triangle indicates only cells adjacent to the P neurons killed. B, A1 and A2 axons cut; P1, P2, and P3 neurons killed. C, A1 and A2 axons cut. D, P1, P2, and P3 neurons killed. The three points indicated by triangles represent preparations in which the Gs were advancing anteriorly and laterally in anomalous axon tracts. E, P2 and P3 neurons killed. F, P1 neuron killed.

easily accomplished without damaging the remaining cells. Great care was taken to limit the amount of time the P cells were exposed to fluorescent illumination so that the unkilld, dye-coupled P cells would not be photodamaged. Killing only the P2 and P3 neurons appears to produce a result similar to the elimination of all three P neurons (Fig. 5E). Killing the P1 neuron alone has no observable effect (Fig. 5F).

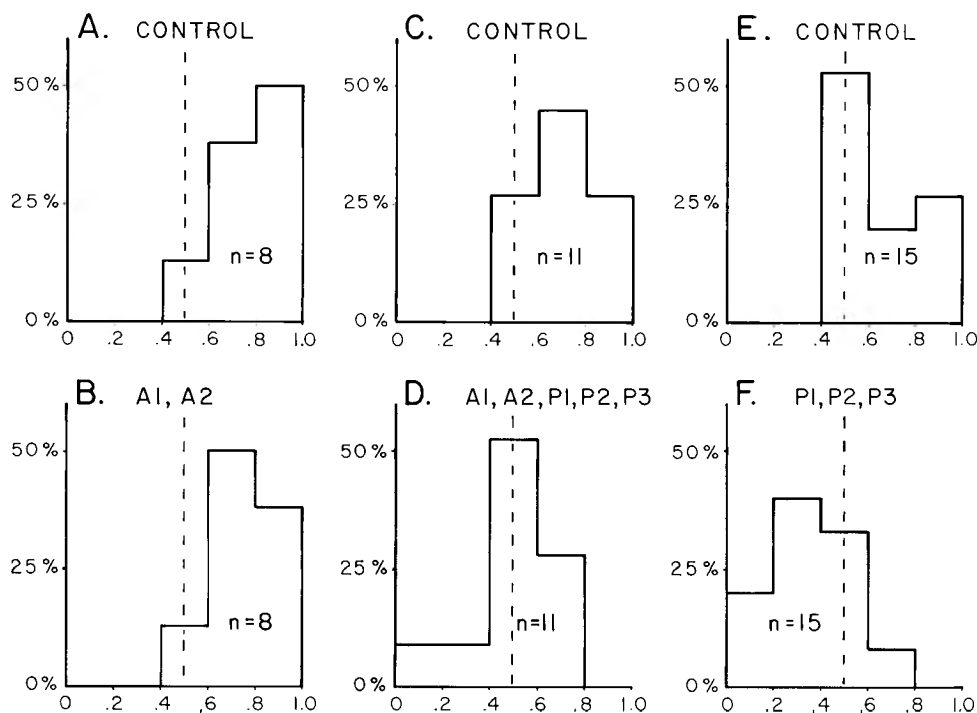
**Effects of axon ablations on the polarity of G's growth.** *In ovo*, G's primary growth cone extends in an exclusively anterior direction. The growth cone of its twin sibling, the C neuron, first branches symmetrically and then extends posteriorly. Therefore, we were curious as to whether the A and/or P axons might play a role in signaling not only the location but also the direction in which G's growth cone should advance. This issue has been difficult to approach because G's behavior in culture is not entirely normal in this respect. It is common for cultured G neurons to have a significant posteriorly directed neurite. For this reason it was necessary to devise a rough measure of G's tendency to grow anteriorly as opposed to posteriorly. Therefore, we compared the distance between the choice point and the tip of G's anterior neurite to the distance between the tip of G's most posterior neurite to the tip of its most anterior neurite. This ratio will be equal to 1 if G extends only anteriorly, 0 if it extends only posteriorly, and 0.5 if it branches symmetrically.

Figure 6 is composed of histograms which relate the frequency of this measurement to several experimental conditions and their matched control groups. The control Gs from eight embryos in which the A1 and A2 axons were severed extend in a predominantly anterior direction (Fig. 6A). The G neurons on the experimental sides of the same embryos also extend in a predominantly anterior direction (Fig. 6B). The control Gs from 11 embryos in which the A1, A2, P1, P2, and P3 axons were ablated extend in a predominantly anterior direction (Fig.

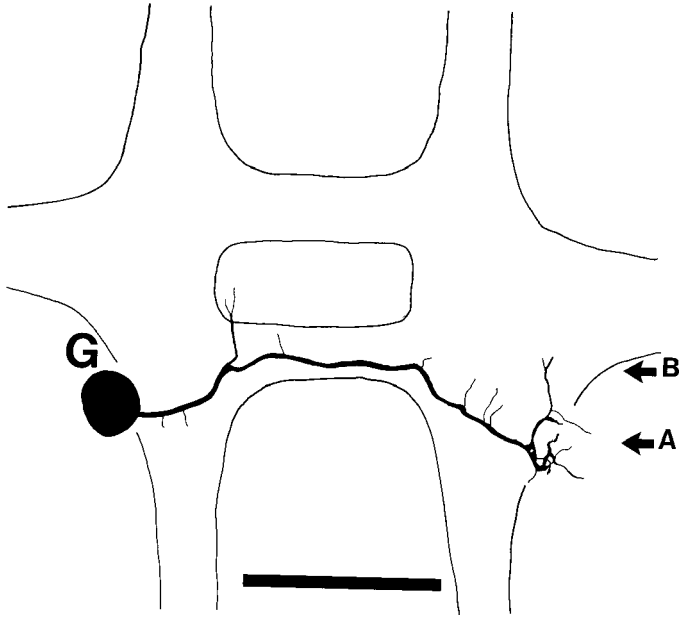
6C). In contrast, the experimental Gs from the same embryos have processes which are more symmetrically distributed (Fig. 6D). The control Gs from another group of 15 embryos in which only the P1, P2, and P3 neurons were killed extend in a predominantly anterior direction (Fig. 6E). The G cells on the experimental sides of the same embryos extend in a predominantly more posterior direction (Fig. 6F). In neither the group in which just the A axons were ablated nor the group in which the A and P axons were ablated did we find any tendency for the experimental Gs to grow further posteriorly than the control Gs (data not shown). In the group in which only the Ps were ablated, although there is a change in the ratio of anterior to posterior extension (Fig. 6F), there is only a slight tendency for the experimental G to extend further posteriorly than the control G. Thus, most of the difference in the ratio is because the experiment G does not extend as far anterior as the control G.

#### Ultrastructural observations of experimental embryos

**Effects of ablating the A and P axons.** In a previous section we described our light level observations of the effects of ablating the A and P axons on the G growth cone. In such experimental embryos, the G cell behaved abnormally in that its growth cone branched extensively and did not extend as far anteriorly as normal. However, we were interested in whether the G growth cone branched in the proper location and, moreover, whether it showed a high affinity for any other particular axons in the absence of the A and P axons. To answer these questions, we examined one of these preparation in the transmission electron microscope after injection of the G neuron with HRP. The light level morphology of this G neuron is shown in Figure 7. As viewed two dimensionally, this G growth cone branched extensively, did not extend very far anteriorly, and extended further laterally than normal.



**Figure 6.** Polarity of G's growth on the control and manipulated sides of cultured embryos. The measurement on the X axis represents the proportion of G's extension in the anterior-posterior axis which was directed anteriorly; the Y axis represents the frequency of observations within the group. In one group the A1 and A2 axons were cut. A, Control side response. B, Experimental side response. In another group the A1 and A2 axons were cut; and the P1, P2, and P3 neurons were killed. C, Control side response. D, Experimental side response. In a final group the P1, P2, and P3 neurons were killed. E, Control side response. F, Experimental side response.



**Figure 7.** Camera lucida drawing of the G neuron in an experimental embryo in which the A and P axons were ablated. The G neuron shown in this figure was filled with HRP, fixed and embedded for TEM, and subsequently semiserially sectioned. Figure 8 shows two representative sections taken at levels A and B as shown in this figure. Note that the G growth cone behaves abnormally, branching extensively and extending further lateral than normal. Calibration bar: 50  $\mu$ m.

We examined this preparation in semiserial sections (1- $\mu$ m intervals). Two representative sections are shown in Figure 8, taken from levels A and B as shown in Figure 7. The neuropil developed considerably in culture, as seen by the increased number of axon fascicles (compare Fig. 8 to Fig. 3A in the companion paper (Bastiani et al., 1984) which shows the neuropil at the stage the ablations were made). The A/P fascicle did develop (see *curved arrows* in Fig. 8), even in the absence of the A and P axons. We judge this bundle to be the A/P fascicle by its location and the position and identity of surrounding fascicles as compared to the control side. Evidently some other growth cones, not including G, fasciculated with one another to form the A/P fascicle in the developing neuropil (normally, other axons in addition to G continue to join the A/P fascicle after the As and Ps). However, the G growth cone behaved abnormally as it extended laterally past the location of the A/P fascicle. The G growth cone extended further laterally and ventrally and branched extensively (see *straight arrows* in Fig. 8). In a semiserial reconstruction, none of its many branches or filopodia showed a high affinity for any particular axon fascicle in the neuropil.

In this example, the G cell extended one lateral growth cone along the axon of the contralateral G and C neurons. Normally, it gets off of the contralateral G and C axons when it climbs onto the A/P fascicle. In this experimental embryo, however, this lateral growth cone extended toward and around the G and C cell bodies. The high affinity shown by the G growth cone for the contralateral G and C cell bodies was seen in several other experimental embryos and is illustrated from another experimental embryo (P axon ablations) as shown in Figure 13.

**Effects of ablating only the P axons.** Our light level observations showed that ablation of only the P cells does affect G's morphology. In Figure 9 is shown a two-dimensional light level drawing of a preparation prepared for electron microscopy. In this preparation, both the G and C neurons were filled with HRP. Both growth cones branched abnormally on the experimental side, the G growth cone made considerably less progress anteriorly than normal, and both growth cones extended lat-

erally and appeared to wrap around the C and G cell bodies. To ascertain the exact location, extent, and affinity of the branches of the G and C growth cones in this preparation, we examined the HRP-filled profiles in semiserial sections in the electron microscope. The sections shown in Figures 10 to 13 are taken from positions 1 (left and 1 to 4 (right) shown in Figure 9 as follows: Figure 10, 1 left (control side); Figure 11, 1 right (experimental side); Figure 12, A and B, positions 3 and 4, respectively; Figure 13, two different magnifications at position 2 through the C cell body.

On the control side, the A/P fascicle formed in the normal location (Fig. 10A), and the A and P axons were joined by several other axons in addition to G (Fig. 10B). On the experimental side, the A/P fascicle also formed in the normal location (Fig. 11A), and here, too, several other axons joined the A axons in the absence of the P axons. However, the G axon was not one of them. Rather, the G growth cone extended just ventral to the A/P fascicle as normal, but instead of extending dorsally and wrapping around the P axons as normal, it continued to grow and branch extensively in the lateral and ventral portions of the neuropil in an abnormal fashion (Fig. 11A). When the most anteriorly directed branches of the G growth cone were followed in semiserial sections, they were found located in abnormal locations in the ventral and lateral neuropil. Furthermore, they were found not to be tightly associated with any particular axon bundle.

The most prominent branches of G's and C's growth cones extended laterally along the axons of the contralateral G and C neurons and then wrapped around their cell bodies (Fig. 13A). The many small branches and filopodia of the G and C growth cones showed a very high affinity for the surfaces of the C and G cell bodies (Fig. 13B).

Of the several experimental embryos prepared for electron microscopy in which the P axons were ablated, the preparation shown in Figures 9 to 13 appeared quite typical: the G growth cone behaved abnormally. However, we also examined in the TEM that preparation in which the G growth cone behaved most normally in the absence of the P axons, as shown in Figures 14 and 15. At the light level, the G growth cone in this preparation extended further anteriorly than in other experimental embryos, although it also extended further laterally than normal. It is likely that this G growth cone did not extend as far anteriorly as a control G, but since we did not fill the G neuron on the control side of this embryo, we cannot be certain.

Examination of this experimental embryo in semiserial sections in the electron microscope led to the following observations. First, the A/P fascicle formed in the normal location, but it was not as tight as *in ovo*. Rather, it appeared to fractionate into smaller bundles (Fig. 15B) which were sometimes held together as a tighter single bundle (Fig. 15A). The major branch of the G growth cone did follow this A/P fascicle, although the tip of the G growth cone was not firmly apposed to any axon within the bundle as it normally is, and its filopodia did not show a particularly high affinity for the axons within the bundle (Fig. 15B). Rather, the tip of the G growth cone was suspended in space and did not appear to have grown as far anteriorly as normal. However, whereas in some experimental embryos the G growth cone extended laterally past the A/P fascicle in the absence of the P axons, in this case it clearly showed some preference for the fascicle even in their absence. It is possible that this represents an embryo in which one of the P axons was not ablated. Alternatively, the G growth cone may have shown an affinity, albeit less than normal, for either the A axons or some other axons which had joined the bundle.

## Discussion

Our previous descriptive studies showed that the G growth cone and those of its NB 7-4 siblings extend in very close apposition to other specific axons in the developing neuropil



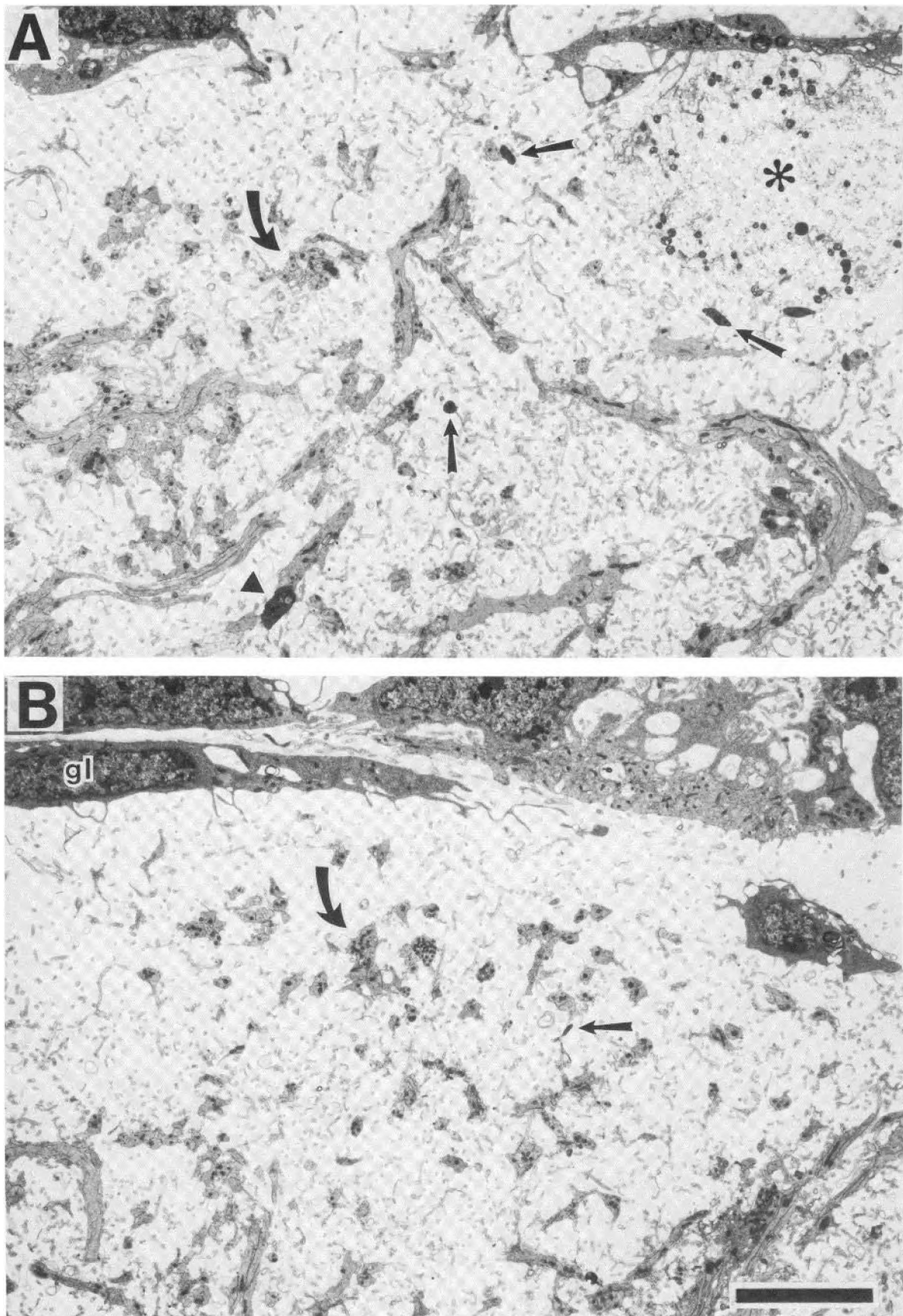


Figure 8. Transmission electron micrographs of the experimental embryo shown in Figure 7 in which the A and P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. A, Section at level A shown in Figure 7. B, Section at level B. The A/P fascicle forms in the absence of the A and P axons (*curved arrow*). The HRP-filled profiles of the G growth cone are shown by the *straight arrows* and *arrowheads*. Note that the G growth cone branches extensively in the ventral (below) and lateral (right) neuropil. The *asterisk* marks the profile of a dying cell; *gl*, glial cell. Calibration bar: A and B, 10  $\mu$ m.

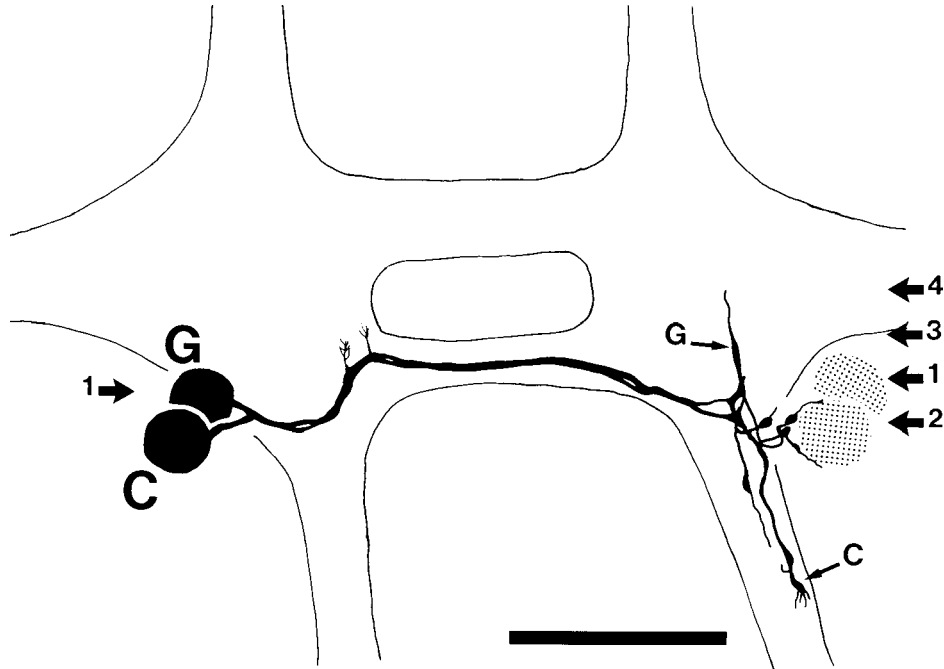


Figure 9. Camera lucida drawing of the G and C neurons in an experimental embryo in which only the P axons were ablated. The G and C neurons shown in this figure was filled with HRP, fixed and embedded for TEM, and subsequently semiserially sectioned. Figures 10 to 13 show representative sections taken at levels 1 on the control (left) side and levels 1 to 4 on the experimental (right) side as follows: Figure 10, level 1 left; Figure 11, level 1 right; Figure 12, A and B, levels 3 and 4, respectively; Figure 13, level 2 at two different magnifications. Note that the G growth cone behaves abnormally, branching extensively and extending further lateral than normal. The lateral branches of the growth cone appear to wrap around the cell bodies of the contralateral C and G neurons. Calibration bar: 50  $\mu$ m.

(Raper et al., 1983b; Bastiani et al., 1984). These observations on selective fasciculation during neuronal development led us to propose the "labeled pathways" hypothesis which extends upon earlier hypotheses, as discussed previously (Goodman et al., 1982; Raper et al., 1983a, b, c). It proposes that early differentiating neurons pioneer a stereotyped array of axonal pathways, that these pathways are differentially labeled, and that the growth cones of later differentiating neurons choose between and extend upon these labeled pathways. This hypothesis predicts that G's growth cone traverses its very precise route in the developing neuropil by first recognizing and then extending along (i) the axons of its sibling Q1 and Q2 neurons, and subsequently its contralateral homologue G and C axons in the Q1 commissural fascicle; and then (ii) the axons of the A1, A2, P1, P2, and P3 neurons in the longitudinal A/P fascicle. Alternatively, G's growth cone could be guided through the neuropil by cues extrinsic to the axons of these neurons and might, therefore, use these axons merely as a convenient mechanical substrate. If the labeled pathways hypothesis is correct, we anticipated that ablating the A and P axons should prevent G from locating and extending anteriorly in its proper location in the contralateral neuropil. If the hypothesis is incorrect, we expected that G's morphogenesis would be unaffected by the absence of the A and P axons.

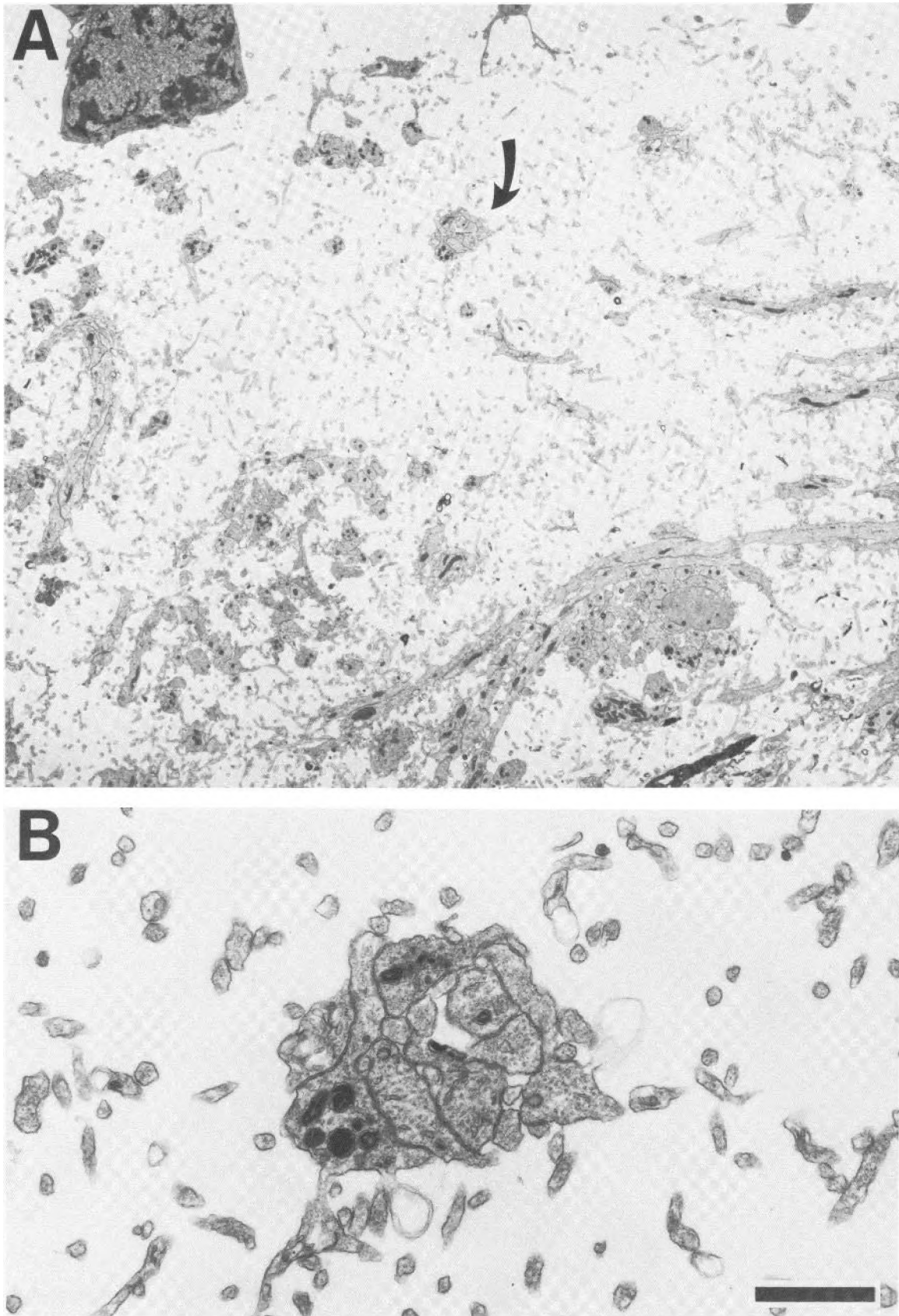
Ablating the A1, A2, P1, P2, and P3 axons at the developmental stage employed in this study did not prevent G from branching in the contralateral neuropil along the anterior-posterior axis. This suggests that the proclivity of G's growth cone to branch in this way is not dictated in an obligatory way by the presence of the A or P axons. In some preparations the G growth cone appeared at the light level to branch in its correct location. However, electron microscopic observations of such experimental embryos in which the A and P axons were

ablated indicated that all of these anterior-posterior branches of the G growth cone were in abnormal locations, usually far ventral, and often further lateral, to the normal location of the A/P fascicle. In some preparations viewed at the light level, multiple anteriorly or posteriorly directed branches were clearly observed in abnormal locations (e.g., Figs. 4, B, C, and E, 7, and 9). In other preparations, a single anteriorly directed neurite extended along an anomalous route (points represented by triangles in Fig. 5D).

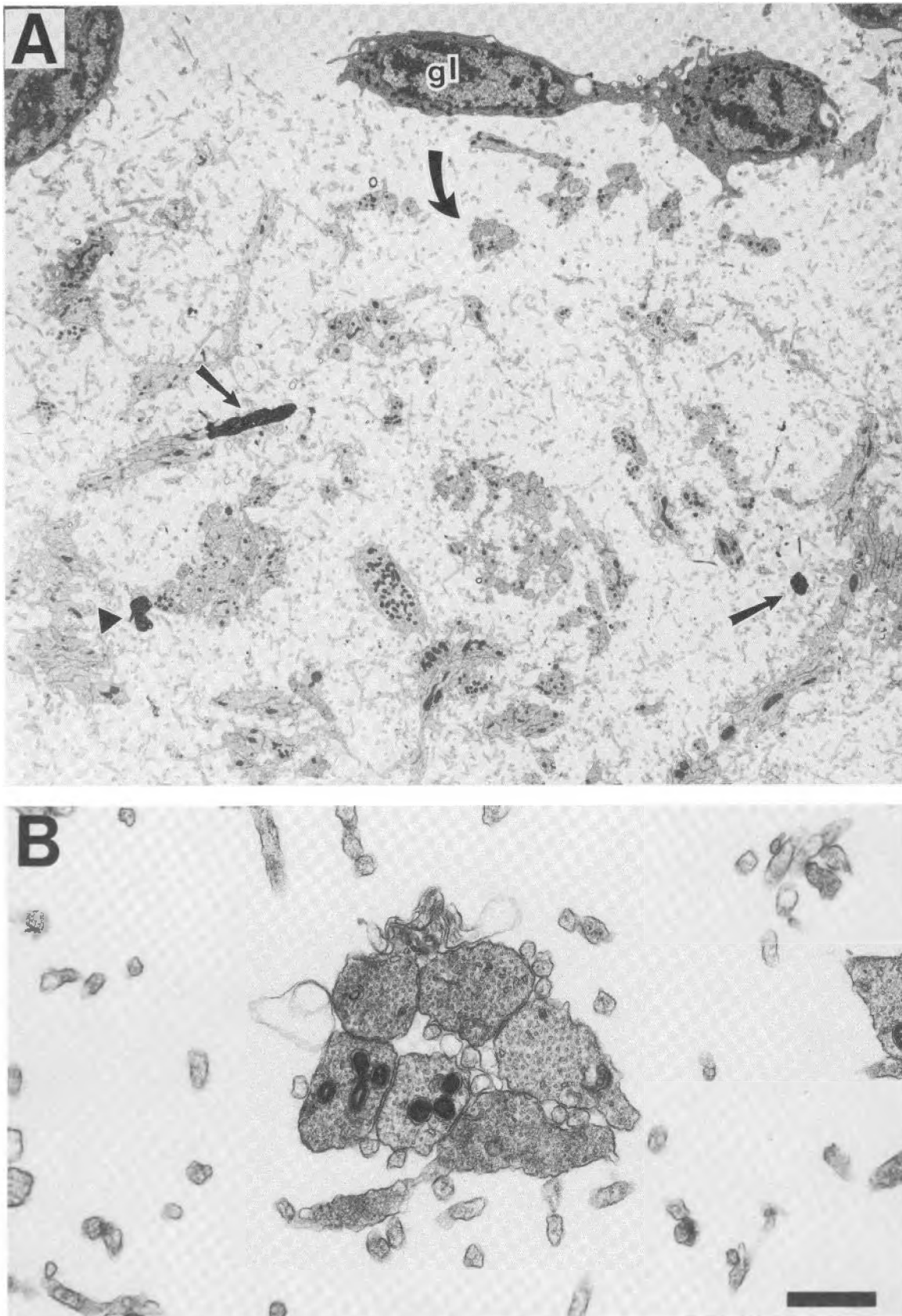
Ablating the A1, A2, P1, P2, and P3 axons clearly inhibits G's ability to extend anteriorly in the contralateral neuropil. In particular, such ablations inhibit G's ability to extend in the proper location in the neuropil. In their absence, G's growth cone shows several abnormal behaviors, typically branching extensively and extending further laterally along the axons of the contralateral G and C neurons, often ending up wrapping around their cell bodies. This implies that these axons, or a subset of these axons, are required for G's normal anterior elongation.

However, this result by itself does not distinguish a permissive from an instructive role for the A and P axons in the extension of G's growth cone. Growth cones require a relatively continuous substrate upon which to crawl (Harrison, 1910). The role of the A and P axons could be to provide such a suitable substrate for the G growth cone. G might then choose to crawl on the A and P axons not because of their intrinsic merits, but because they are the only available continuous substrate compatible with other extrinsic cues determining G's path.

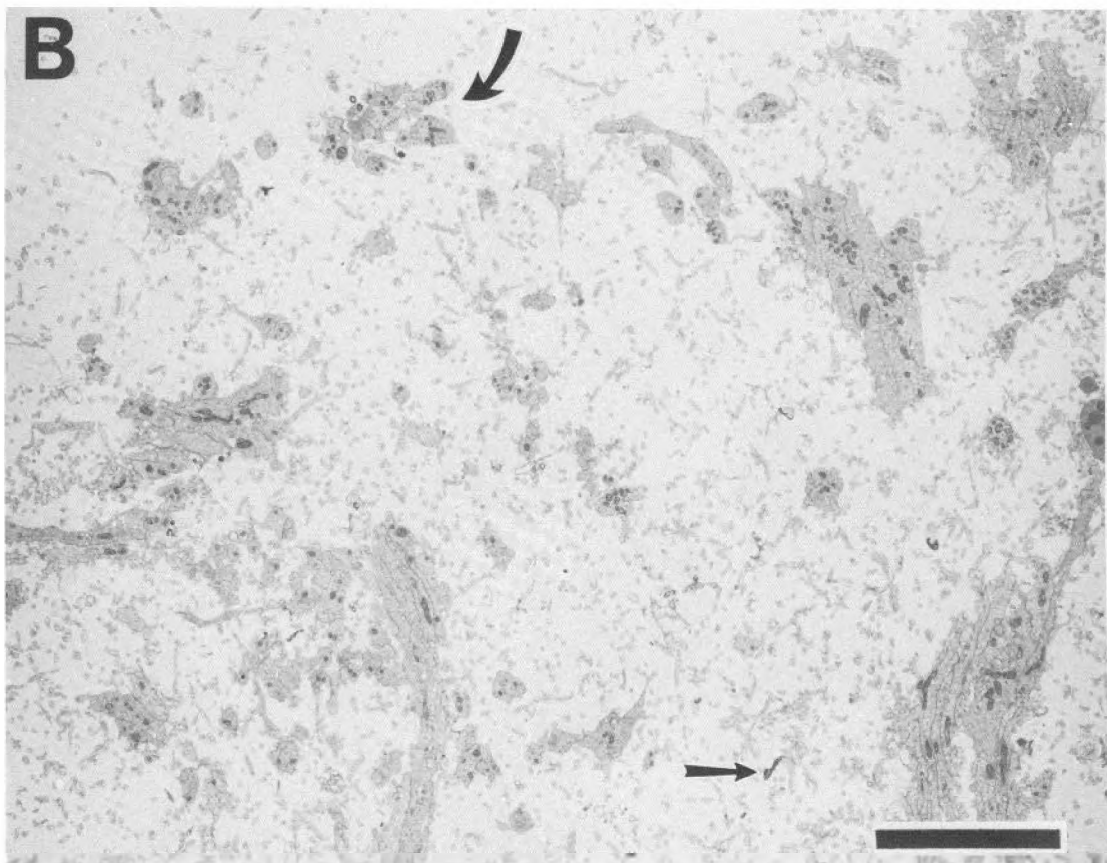
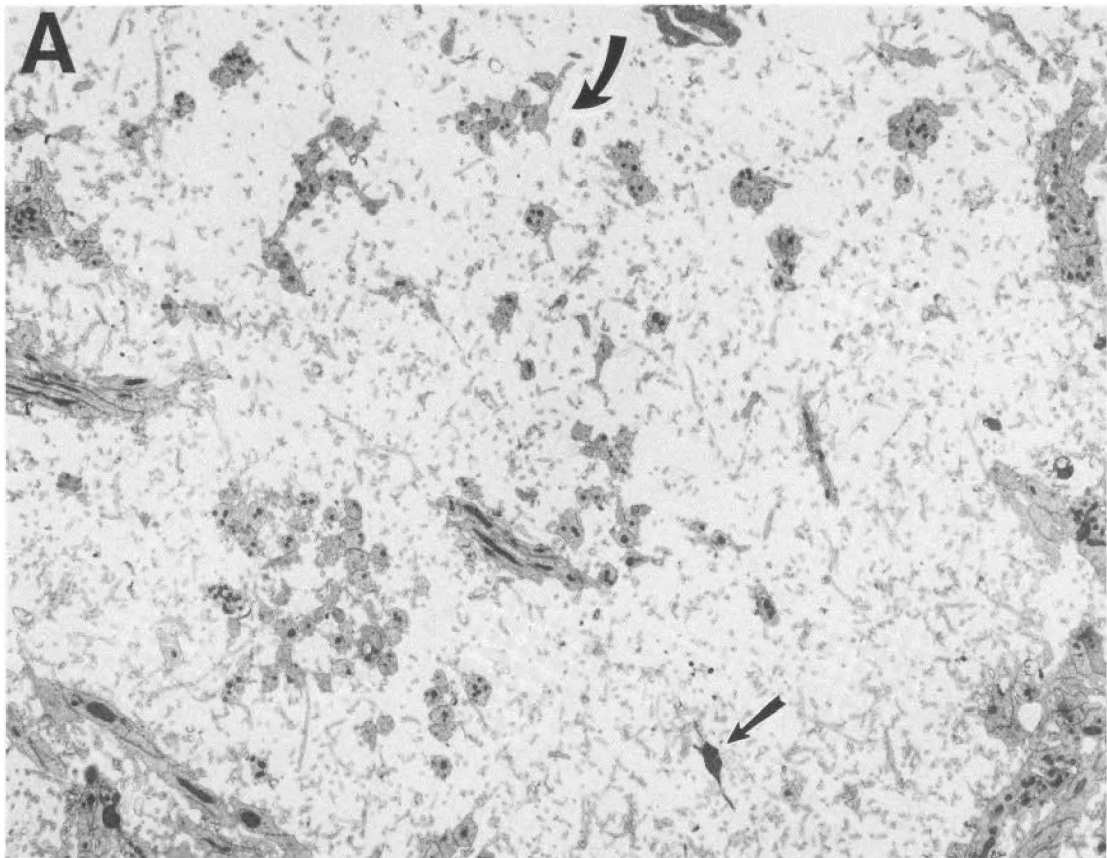
However, other axon fascicles less than 10  $\mu$ m away from the A and P axons traverse a parallel route through the ganglionic neuropil around the time when G elongates anteriorly (e.g., the D fascicle as described in Bastiani et al., 1984). This distance



**Figure 10.** Transmission electron micrographs of the experimental embryo shown in Figure 9 in which only the P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. Section showing the control side (*A*) and A/P fascicle (*B*) at level *l* left as shown in Figure 9. *A*, Low magnification picture of developing neuropil on control side of experimental embryo. The curved arrow shows the control A/P fascicle. *B*, Higher magnification picture of the A/P fascicle on the control side, showing the normal development of a tight bundle in culture. Calibration bar: *A*, 10  $\mu$ m; *B*, 2  $\mu$ m.



*Figure 11.* Transmission electron micrographs of the experimental embryo shown in Figure 9 in which only the P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. Section showing the experimental side (A) and A/P fascicle (B) at level 1 right as shown in Figure 9. A, Low magnification picture of developing neuropil on experimental side, showing the normal development of a tight bundle in culture in the absence of the P axons. Calibration bar: A, 10  $\mu\text{m}$ ; B, 2  $\mu\text{m}$ .



**Figure 12.** Transmission electron micrographs of the experimental embryo shown in Figure 9 in which only the P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. Sections showing the anteriormost branches of the G growth cone on the experimental side at two locations (*A* and *B*) as shown by levels 3 and 4 in Figure 9. *A*, Low magnification picture of developing neuropil on experimental side of embryo. The *curved arrow* shows the A/P fascicle. The *straight arrow* shows the major anterior branch of the G growth cone far ventral to the normal location. *B*, Section further anterior to *A* showing the A/P fascicle (*curved arrow*) and the tip of the G growth cone (*straight arrow*) far ventral and suspended out in space. The G growth cone does not show a high affinity for any particular longitudinal axon bundle in the developing neuropil in the absence of the P axons. Calibration bar: *A* and *B*, 10  $\mu$ m.

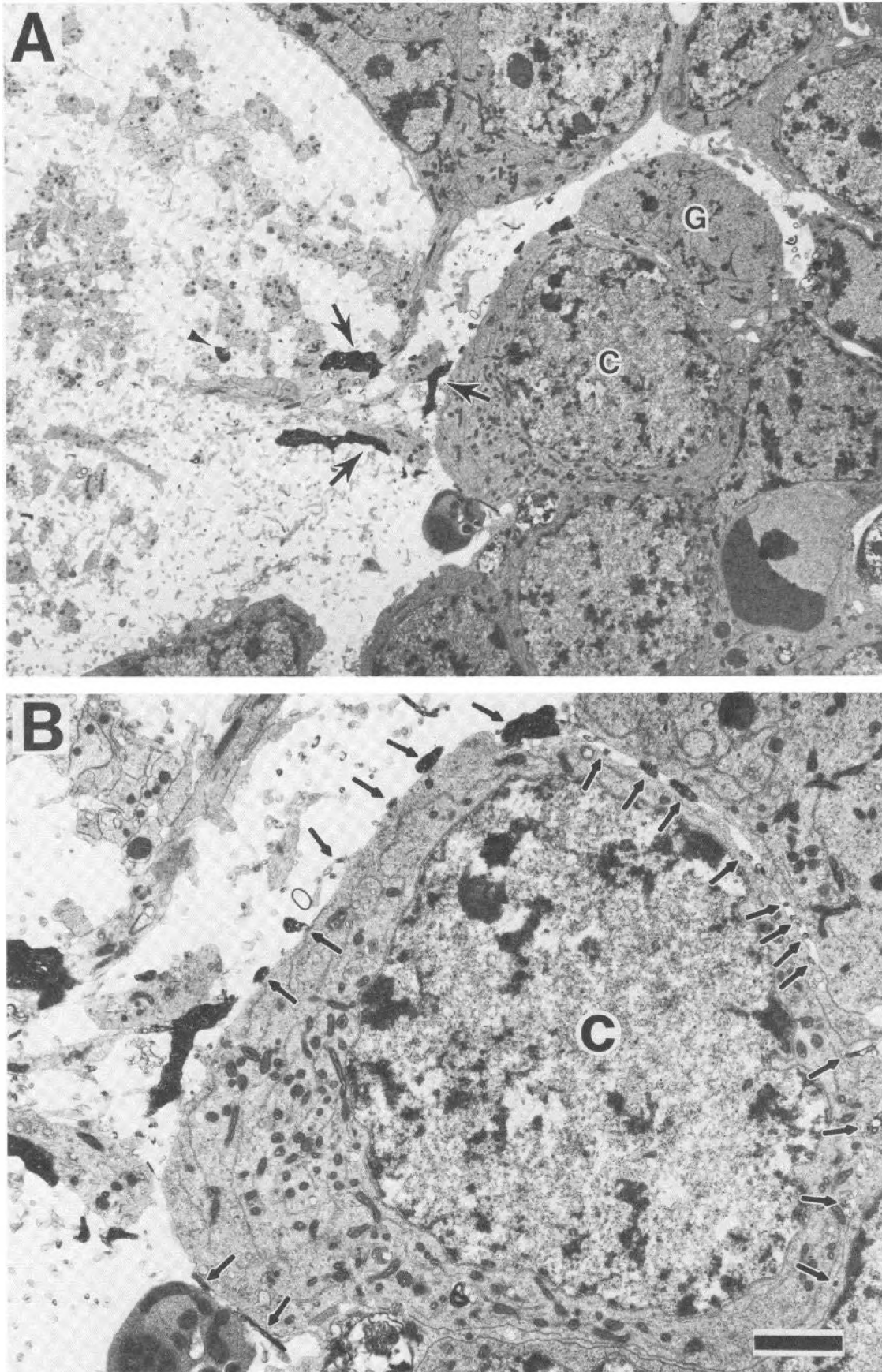
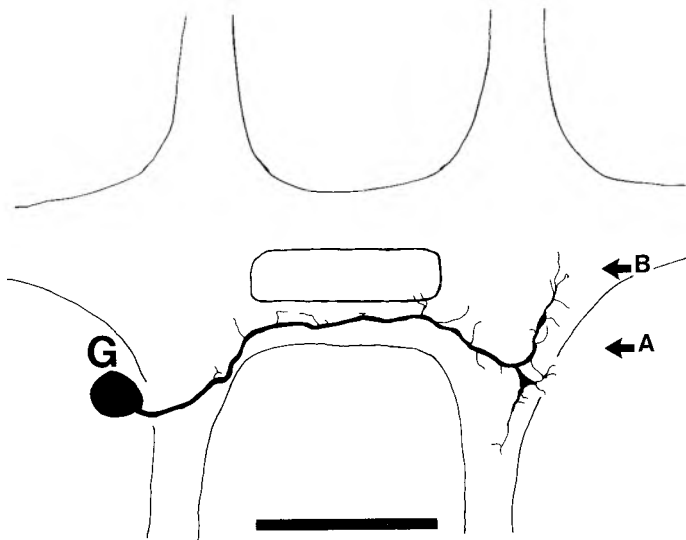


Figure 13. Transmission electron micrographs of the experimental embryo shown in Figure 9 in which only the P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. Sections (at level 2, Fig. 9) showing the lateral branches of the G and C growth cones on the experimental side at two magnifications. A, Branches of both growth cones continued to extend laterally along the axons of the contralateral G and C neurons. B, At higher magnification, the fine branches and filopodia of the G and C growth cones can be seen wrapping around the C (and G, not shown) cell body, showing a high affinity for its surface. Calibration bar: A, 5  $\mu$ m; B, 2  $\mu$ m.



**Figure 14.** Camera lucida drawing of the G neuron in an experimental embryo in which only the P axons were ablated. The G neuron shown in this figure was filled with HRP, fixed and embedded for TEM, and subsequently semiseriably sectioned. Figure 15 shows two representative sections taken at levels A and B. This G neuron represents the most normal behavior seen in culture after ablation of the P axons. The G growth cone extended anteriorly, although probably not as far as normal. Furthermore, it branched extensively and grew further laterally than normal. Calibration bar: 50  $\mu$ m.

is small compared to the distance spanned by G's filopodia at the choice point (Raper et al., 1983a). These alternate fascicles are in place in manipulated embryos, but the G growth cone fails to elongate upon them. Therefore, the A and P axons do not provide the only continuous substrate compatible with G's route through the neuropil. This suggests that the A and P axons play an active, rather than passive, role in G's anterior extension.

Thus, experimental evidence points toward an active role for the A/P fascicle in G's guidance versus the other approximately 25 longitudinal axon fascicles and, moreover, toward an active role for specific axons within the A/P fascicle. If the fascicle plays only a passive role, merely providing a continuous substrate upon which G's growth cone may advance, then the presence of any subset of A and P axons should be compatible with G's normal morphogenesis. However, G behaves differently when different subsets of axons within the A/P fascicle are absent. Ablation of only the A1 and A2 axons does not affect G's normal extension anteriorly. However, ablation of only the P1, P2, and P3 axons does affect G's behavior. Even when the A axons have traversed the lateral neuropil in the absence of the P axons, G's growth cone usually does not advance forward upon them. The differential effects produced by these manipulations suggest that G's growth cone prefers to elongate upon the P axons as opposed to the A axons. This interpretation is strengthened by the observation in unmanipulated embryos that the tip of G's growth cone is found in direct apposition to the P and not the A axons (Bastiani et al., 1984). Thus, G's growth cone behaves as if it can distinguish between the axons of the P and the A neurons. This implies that there are heterogeneous labels within the A/P fascicle itself.

Although the A axons are not necessary for G's anterior extension, neither do they appear to be irrelevant. Ablating the A1, A2, P1, P2, and P3 axons has a more consistent inhibitory effect on G's anterior extension than ablating the P1, P2, and P3 axons alone. However, it is not known whether the less consistent inhibitory effect on G's behavior in the presence of

the A axons is due to G having a partial affinity for them or, alternatively, to G having an affinity for some other axons which subsequently fasciculate upon the A axons.

Hoping to fractionate further the activity of the P cells, we also ablated only the P1 cell or only the P2 and P3 cells. Ablating the P1 cell has no effect on G's ability to extend anteriorly. Ablating the P2 and P3 cells prevents G from extending normally in some preparations but not others. Thus, the ablation of the P1 cell has no effect, the ablation of the P2 and P3 cells has some effect, the ablation of the P1, P2, and P3 cells has approximately the same effect, and the ablation of the A1, A2, P1, P2, and P3 cells has the greatest and most consistent effect. This is suggestive of a dose-related response for these ablations, since as more A and P axons are ablated, the more G's behavior is influenced. This continuum of effects provides an important caveat to the conclusion that the P and the A cells are differentially recognized by the growth cone of the G cell. G's morphogenesis may be more sensitive to the ablation of the P cells since there are three P axons and only two A axons. However, it seems very likely that G differentiates between the A and P axons since the ablation of the P2 and P3 cells has a quite detectable effect on G's behavior, while ablation of the A1 and A2 axons does not.

Finally, we were interested in examining the effects of these ablations on the polarity of G's extension in the anterior-posterior axis. Even if the growth cone of the G cell recognizes its correct location in the neuropil by the labels on axons in its immediate environment, the polarity of its extension along axon pathways could be determined by other mechanisms. *In ovo*, G's primary growth cone turns and extends in an exclusively anterior direction. In unmanipulated cultured embryos, G extends in a predominantly anterior direction but generally has a shorter posteriorly directed neurite as well. Ablation of the A1 and A2 axons has no effect on the proportion of G's growth which is directed anteriorly. Ablation of (i) the A1 and A2 axons, together with the P1, P2, and P3 cells, or (ii) only the P1, P2, and P3 cells leads to G cells that have a greater proportion of their growth in the posterior direction. However, this apparent change in polarity probably comes about by default. The G growth cones on the experimental sides show no significant tendency to extend further posteriorly than their matched controls.

These results are of little help in determining whether G normally uses cues intrinsic or extrinsic to the A and P axons in determining which direction to grow. Without a suitable substrate upon which to elongate anteriorly (e.g., the P axons), G's ability to express a polarity preference is suspect. Instead, these measurements are simply another indication that the P axons are required for G's normal extension anteriorly. However, they do suggest that the distribution of appropriate axonal substrates for elongation could control the polarity of G's extension. G may turn anteriorly because its most preferred substrate is the P axons, and at the time when G's growth cone is making its choice, the P axons are just anterior to it.

In conclusion, our results show that the A and P axons are required for the normal behavior of the G growth cone, and they further suggest that the A and P axons are differentially labeled. The G growth cone appears to prefer the A/P fascicle over all other longitudinal fascicles within the contralateral neuropil and, moreover, appears to prefer the P axons over the A axons within this fascicle. The results from these simple ablation experiments are highly consistent with the labeled pathways hypothesis. At the cellular level, however, further proof for the hypothesis could best be accomplished by manipulations in which the behavior of G's growth cone is observed after altering the locations, rather than simply ablating, the A and P axons in an otherwise intact neuropil.

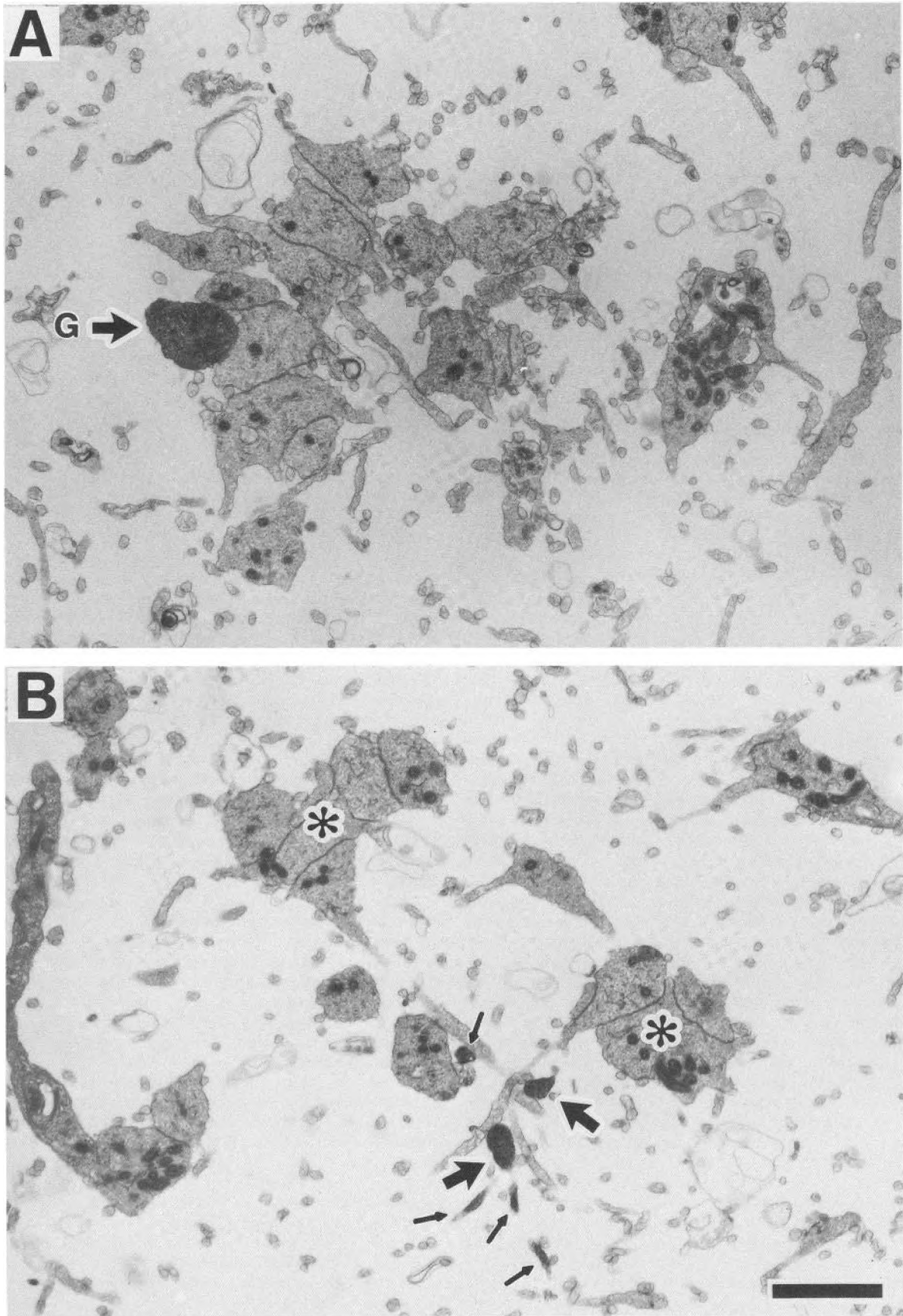


Figure 15. Transmission electron micrographs of the experimental embryo shown in Figure 14 in which only the P axons were ablated and the embryo was subsequently cultured for 40 to 48 hr. Sections showing two differential levels (A and B in Fig. 14) on the experimental side. A, The G growth cone extends anteriorly in the A/P fascicle. B, The tip of the G growth cone (*large arrows*) is not tightly apposed to any particular axon, and its filopodia (*smaller arrows*) do not show a high affinity for any particular axon in the absence of the P axons. The A/P fascicle in this experimental embryo subdivided into smaller bundles, as in B, which sometimes formed a single tighter bundle, as in A. Calibration bar: A and B, 2  $\mu$ m.



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