Guidance of Neuronal Growth Cones:
Selective Fasciculation in the Grasshopper Embryo

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One of the central questions of developmental neuro-biology concerns how the diversity and specificity of individual neurons are generated during embryonic development. One major component of neuronal diversity is the complex axonal morphology of individual neurons, largely generated early in development and intimately involved in the ability of neurons to find their correct synaptic targets. By a process of precise path-finding, growth cones find the appropriate neurons or muscle cells, often by traveling long distances along stereotyped routes that involve a series of cell-specific choices and turns.

Growth cone motility, as described from cell culture experiments, involves three phases: extension, adhesion, and contraction (Bray 1982; Letourneau 1982). Growth cones extend numerous fingerlike filopodia, approximately 0.1 μm in diameter and up to 50 μm or more in length. These filopodia radiate in many directions from the growth cone, transiently exploring their environment. Some of the filopodia contact other cell surfaces or extracellular basement membranes; they strongly adhere to some of these surfaces but their adhesion to others is much weaker. Filopodia are retracted in a contractile cycle. If adhesion is weak, the filopodium is retracted; if, however, its adhesion is strong, then tension in that direction is increased during the contractile cycle and the leading tip of the growth cone advances toward the point of attachment (Bray 1982; Letourneau 1982). Thus, the key to understanding the diverse and specific choices made by growth cones during embryogenesis involves in large part understanding the behavior, environment, and selective adhesion of their filopodia as they make cell-specific decisions.

We would like to understand how the growth cones of different neurons, confronted with the same environment, make different and stereotyped choices. Such divergent choices by growth cones imply both heterogeneity in their cellular environment and heterogeneity in their responses to that environment. We would like to know what cellular and molecular cues in an embryo influence the choices made by individual growth cones, and how these growth cones are determined by their mitotic ancestry and earlier cell interactions to respond to those cues. Our strategy has been first to examine, and then to manipulate, the cellular environment of identified growth cones during embryonic develop-ment. For these studies, we have used the highly accessible and relatively simple CNS of the grasshopper embryo (e.g., Goodman and Spitzer 1979; Goodman and Bate 1981).

In this paper, we review what we have learned about the growth cones of the first six progeny of neuroblast 7-4 in the grasshopper embryo, and in particular focus our attention on the growth cone of the G neuron (Raper et al. 1983a). These growth cones, like most other embryonic growth cones, find themselves in an environment surrounded by the axons of other previously differentiating neurons. These axons run in fascicles that take the form of a scaffold of nearly orthogonal axon bundles (Fig. 1). These growth cones are within filopodial grasp of many different axon bundles, yet each makes a cell-specific choice of which bundle to run in.

Here we show that the G growth cone fasciculates upon a discrete bundle of axons in preference to other nearby bundles (Raper et al. 1983b). This bundle, called the A/P fascicle, is established by the A1, A2, P1, and P2 neurons; the A1 and A2 axons run anteriorly where they meet and fasciculate with the P1 and P2 axons (and shortly thereafter, the P3 axon), which run posteriorly. As G reaches its choice point and subsequently extends anteriorly upon the A/P fascicle, its filopodia are more often in contact with the A/P fascicle than with other nearby axon fascicles (Bastiani et al. 1984). Within the fascicle itself, the tip of G's growth cone is found to be closely associated with the P and not the A axons. These findings suggest that G is able to distinguish the A/P fascicle from other axon bundles, and moreover appears able to distinguish the P axons from the A axons.

Our observations led us to propose the ''labeled pathways'' hypothesis (Goodman et al. 1982; Raper et al. 1983b). It proposes (1) that pioneering neurons establish stereotyped axonal pathways; (2) that these axonal pathways are differentially labeled on their cell surfaces; and (3) that later growth cones are differentially determined in their ability to make specific choices of which labeled pathways to follow. This hypothesis includes the notion that filopodia are actively involved in sampling the surfaces of axon bundles within their grasp and that differential cell adhesion by filopodia and growth cones mediates the selective fasciculation.

We tested this hypothesis by examining the effects of ablating the A1, A2, P1, P2, and P3 axons upon the behavior of the G growth cone (Raper et al. 1984). If

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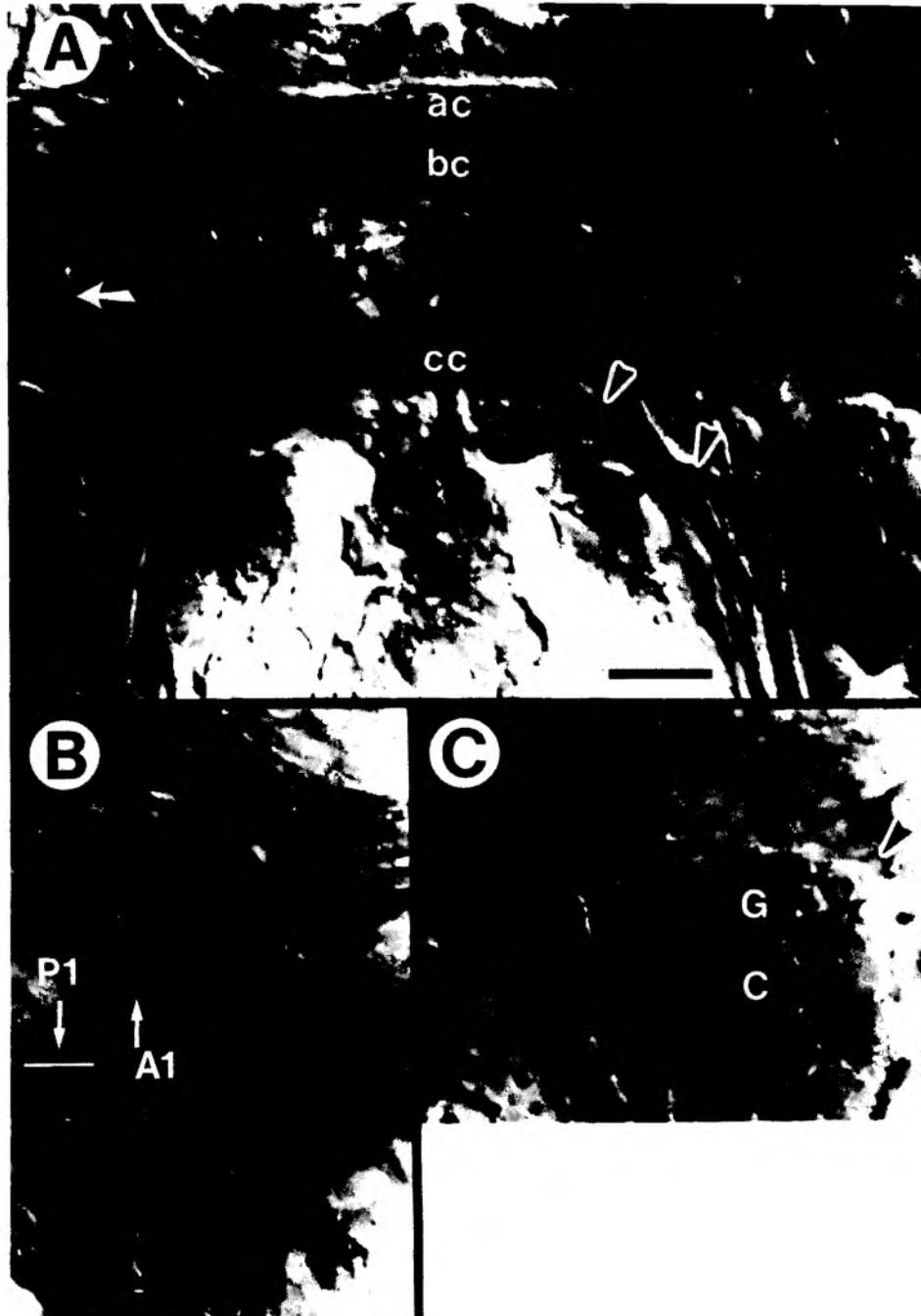
Figure 1. Axonal scaffold of the second thoracic (T2) segment of a whole-mount preparation of a 40% grasshopper embryo, as shown in a photograph of a narrow focal plane of a dorsal view of the neuroepithelium. The preparation is stained with the I-5 monoclonal antibody and an HRP-labeled second antibody (Chang et al. 1983). Only some of the axonal pathways present in the three-dimensional developing neuropil are shown. The age shown is just before the G and C growth cones make their divergent choices. (*A*) One posterior (cc) and two anterior (ac, bc) commissures join the two sides of the ganglion. A pair of longitudinal connectives join each ganglionic segment to the next. The fibers in these connectives spread apart as they enter the neuropil and are gathered together again as they leave. The axon bundle in which G and C cross the posterior commissure is indicated (black arrowheads). The fiber bundle upon which G and C diverge is also shown (white arrow). (*B*) A higher-power view of the A1 and P1 growth cones (see text and Fig. 2) meeting to form the A/P fascicle upon which the G and C growth cones diverge. The directions in which the growth cones are extending are indicated by arrows. The posteriormost tip of the P1 growth cone is indicated by a horizontal line. (*C*) A high-power view of the G and C cell bodies. A dying cell, probably Q1 or Q2, is indicated by the arrowhead. Bar, *A*, 20 μm; *B*, 25 μm; *C*, 30 μm. (Reprinted, with permission, from Raper et al. 1983b.)

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the A/P fascicle specifically guides G's growth cone through the neuropil, then its ablation should prevent G's normal behavior. 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's growth cone and that it is the P axons specifically that appear to be most active in this role. These results, as described in detail below, thus support the "labeled pathways" hypothesis.

Divergent Choices Made by the Growth Cones of Sibling Neurons

The grasshopper embryo is an excellent preparation in which to study the guidance of neuronal growth cones because (1) individual identified neurons and their growth cones are highly visible and accessible, and (2) the pattern of neurons and their axons is relatively simple and highly stereotyped (e.g., Goodman and Spitzer 1979; Goodman et al. 1979, 1981). The cell bodies, axons, and growth cones of individual neurons can be visualized with Nomarski interference contrast optics, penetrated with microelectrodes, and filled with a variety of markers to reveal further their axons, growth cones, and filopodia (e.g, Goodman et al. 1982; Taghert et al. 1982; Raper et al. 1983a). The processes of these cells can be individually identified in the transmission and scanning electron microscopes (e.g., Bastiani et al. 1982, 1984; Bastiani and Goodman 1983; Raper et al. 1983b). Furthermore, many of these cells can be visualized using a variety of monoclonal and serum antibodies as specific probes (e.g., Ho and Goodman 1982; Jan and Jan 1982; Bentley and Keshishian 1982; Taghert et al. 1982; Chang et al. 1983; Ho et al. 1983; Kotrla and Goodman 1983).

To study the guidance of neuronal growth cones during embryonic development, we rely on our ability to identify cells and their growth cones uniquely before they have fully differentiated and thus revealed their complete morphological identity. This is quite possible in the grasshopper embryo because the identified neurons in the CNS arise at precise locations in the family tree from two types of identified precursor cells called neuroblasts (NBs; Bate 1976) and midline precursors (MPs; Bate and Grunewald 1981). Thus, cell lineages can be constructed relating a neuron's precursor and cell division of origin to its differentiated identity (e.g., Bate 1976; Goodman and Spitzer 1979; Bate and Grunewald 1981; Goodman and Bate 1981; Raper et al. 1983a; Taghert and Goodman 1983).

The grasshopper's CNS is segmentally arranged with a chain of cephalic, thoracic, and abdominal ganglia. The second thoracic (T2) ganglion contains about 2000 neurons. Because the neurons largely arise as symmetric bilateral homologs, we can consider the T2 hemiganglion as consisting of about 1000 unique neurons. Each segmental ganglion is generated by a precise segmentally repeated pattern of precursor cells,

containing two bilaterally symmetric plates of 30 NBs, an unpaired median NB (MNB), and seven MPs. Each NB is a stem cell, maintaining its large size as it divides repeatedly to produce a chain of smaller ganglion mother cells. Each ganglion mother cell in the chain divides once more, thus producing a chain of paired ganglion cells that subsequently differentiate into neurons. The two bilaterally symmetric NBs 7-4 each push a string of progeny up toward the dorsal posterior surface of every segment. Because of their location on the dorsal surface of the ganglion, the progeny of NB 7-4 can be visualized easily and are highly accessible to microelectrode penetration throughout their development (Fig. 1).

NB 7-4 gives rise to about 100 progeny. The first ganglion mother cell born from NB 7-4 divides and gives rise to a pair of identified neurons called Q1 and Q2 (Fig. 2). The second ganglion mother cell gives rise to the G and C neurons, and the third ganglion mother cell gives rise to the Q5 and Q6 neurons. Fortunately, the adult morphology and synaptic connectivity of the sibling G and C neurons have been extensively studied in Keir Pearson's laboratory (e.g., Pearson et al. 1980; Pearson and Robertson 1981). Both neurons originally were identified and their morphologies described in the second thoracic (T2) ganglion of the adult grasshopper, and thus our embryonic studies concentrate on the T2 segment. The differences between these two neurons are of utmost behavioral importance; for example, the C neuron is involved in initiating the jump behavior and makes very strong synaptic connections onto extensor and flexor motor neurons of the third thoracic (T3) jumping leg. The G neuron, on the other hand, makes only weak synaptic connection onto the extensor motor neuron, but makes a strong synaptic connection onto the M interneuron which in turn inhibits the flexor motor neurons; the C neuron does not connect with the M neuron. The G neuron also synapses on the C neuron, but not vice versa. The morphologies of the two sibling cells have certain similarities, yet striking differences as well. The large axon of the G neuron runs anteriorly to the brain in the lateral portion of the ventral nerve cord; its small axon (a secondary growth during embryogenesis) runs posteriorly to the next ganglion in a more medial portion of the nerve cord. The large axon of the C neuron runs posteriorly to the T3 segment in the lateral portion of the ventral nerve cord.

How do these cell-specific differences between the G and C neurons arise? Ultimately, we would like to explain how G and C assume their distinctive morphologies and locate their appropriate targets by explaining how their growth cones extend, turn, and branch at specific places in the developing neuropil. The initial morphological development of these neurons is nearly identical. Their growth cones extend upon the same axonal pathway across the posterior commissure of the developing ganglion neuropil, although several axon bundles in this commissure are within filopodial grasp (Figs. 1 and 2). However, after reaching the contralateral side of the neuropil, their growth cones
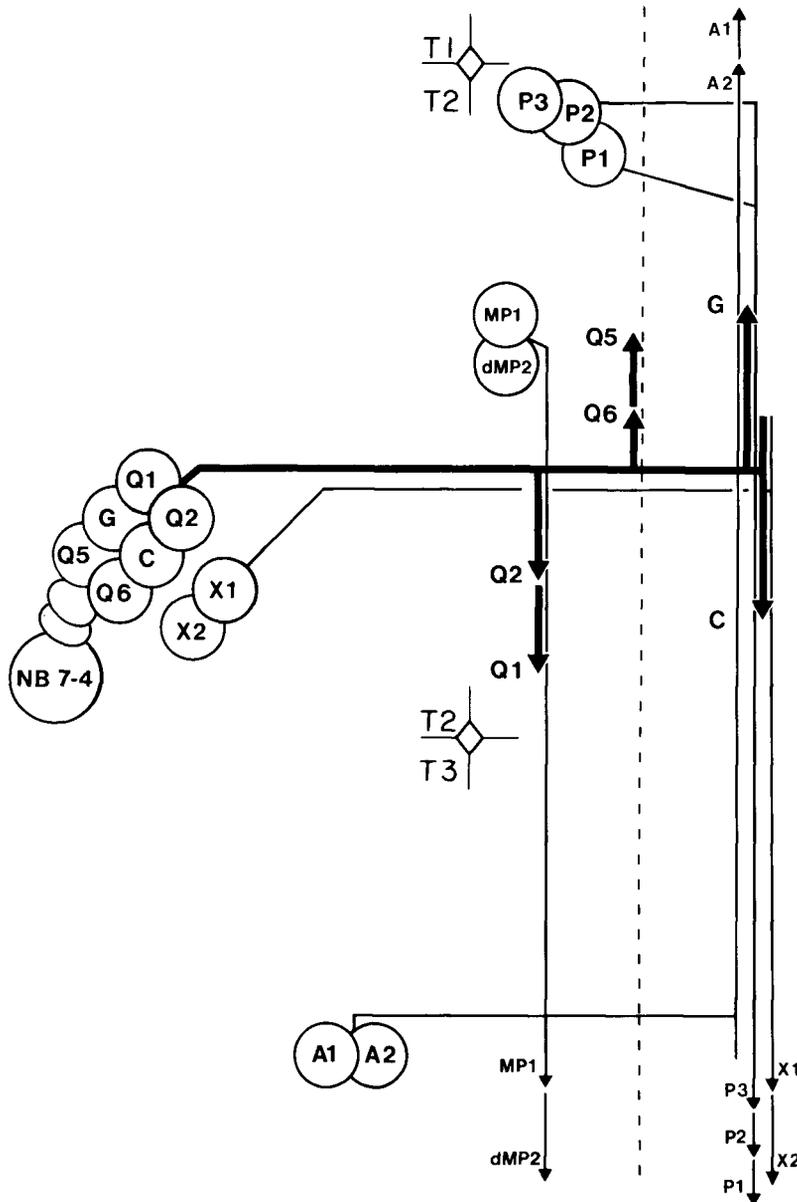
Figure 2. Schematic diagram of the divergent choices made by the growth cones of the first six progeny of NB 7-4. All six growth cones choose the same pathway across the posterior commissure, yet make divergent choices in the contralateral neuropil. Q1 and Q2 turn posteriorly upon the MP1/dMP2 fascicle. G extends anteriorly upon the A/P fascicle. C extends posteriorly in this same axon bundle once other axons (including X1 and X2) have joined the bundle. Q5 and Q6 extend anteriorly in a different, more medial pathway (- - - - -). (Modified from Raper et al. 1983b.)

diverge from each other at stereotyped, cell-specific choice points: Q1 and Q2 turn posteriorly along the most medial axon pathway, G and C turn in opposite directions along the lateral pathway, and Q5 and Q6 turn anterior along a different medial pathway (Fig. 2).

The growth cones of first G and then C extend past the location in the contralateral neuropil where Q1 and Q2 turned posteriorly. G's growth cone continues laterally until it reaches a specific location at the lateral margin of the contralateral neuropil (Fig. 3). Here it often appears to pause for periods of up to 10 hours. During this period in which G's growth cone stops extending laterally, C's growth cone catches up to G's. It

is quite common for the G and C growth cones to have nearly identical morphologies and positions in the neuropil for many hours. Once G's growth cone has begun to extend anteriorly, its rate of growth increases to about 20 μm/hour. This period of rapid elongation leaves C's growth cone behind at the choice point. C's growth cone branches and extends quite slowly in both directions. Its posteriorly directed branch eventually begins to elongate rapidly into the T3 ganglion and beyond.

We observed the filopodia of the G and C growth cones by visualizing cells after intracellular injection of either horseradish peroxidase (HRP) or Lucifer Yellow

A

39-40%

B

40-41%

C

42%
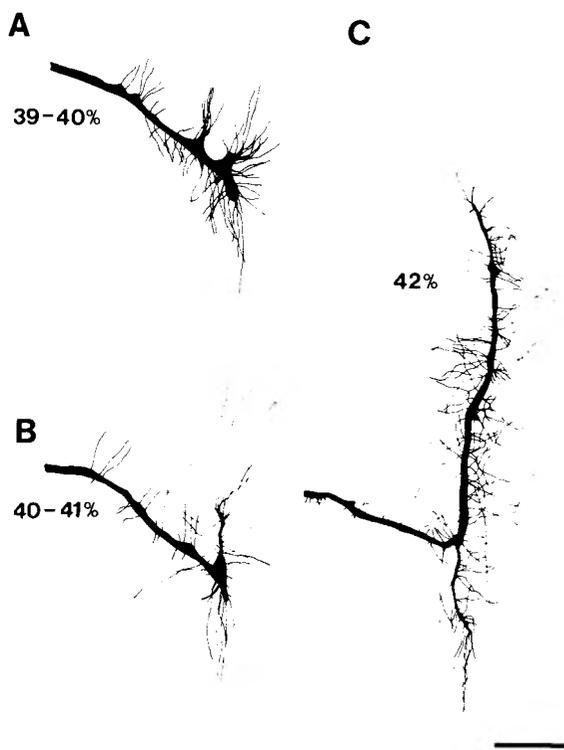
Figure 3. Structure of the G growth cone at different stages during and after its cell-specific choice to extend anteriorly upon the A/P fascicle. (*A*) The morphology of the G growth cone before it turns anteriorly is large and complex. Filopodia radiate in profuse tufts from reproducible locations called active sites at which bumps generally are observed. (*B*) Just as G begins to extend anteriorly, many long filopodia project along or near the path G's growth cone will follow. (*C*) As G proceeds anteriorly, filopodia continue to project along the path it is taking. The growth cone is sometimes tapered, and many lateral filopodia always extend from the newly formed axon behind it. *A* and *B* are camera lucida drawings of neurons filled with Lucifer Yellow and visualized with an anti-Lucifer Yellow antibody reacted with an HRP-labeled second antibody. The cell shown in *C* was filled directly with HRP. Bar, 2 μm. (Reprinted, with permission, from Raper et al. 1983a.)

followed by HRP immunocytochemistry with a serum antibody to Lucifer Yellow (Taghert et al. 1982). The shape of the G growth cone and its filopodia depends on its location in the neuropil (Fig. 3). As the G growth cone reaches its choice point and slows down, it usually becomes quite broad and complex in shape. It sometimes has several anteriorly directed bumps with filopodia extending in tufts from each of these bumps, called active sites (Fig. 3A). The G growth cone becomes long and tapered once its rapid growth into the connective has begun (Fig. 3C). The young axon behind the growth cone generally has numerous lateral filopodia that later disappear.

Selective Fasciculation onto Specific Axonal Pathways

One prominent feature of G's environment at its choice point is the scaffold of nearly orthogonal axon bundles elaborated by previously differentiating neurons (see Fig. 1). As the G growth cone turns anteriorly, its growth cone always fasciculates upon a discrete bundle of axons in preference to other nearby bundles. We were able to identify the A1, A2, P1, and P2 neurons whose axons initially establish this bundle, called the A/P fascicle, first by axonal microelectrode penetrations and dye injections, and subsequently by electron microscopy (Figs. 2 and 4C). The axons of the A1 and A2 neurons extend anteriorly through the dorsal lateral neuropil. They meet and fasciculate upon the two posterior growing axons of the P1 and P2 neurons. The axon of the P3 neuron joins the fascicle just after P1 and P2. The A1 and A2 cell bodies are found in the T3 ganglion, on the side contralateral to their axons. The P1, P2, and P3 cell bodies are found in the T2 ganglion, on the side ipsilateral to their axons. The P1, A1, and A2 growth cones move through G's choice point at about the same time that G turns anteriorly.

C's growth cone extends posteriorly within the same axon bundle as G's. However, by the time C begins to extend predominantly in the posterior direction, there are several more axon profiles in the bundle in addition to the G, C, A1, A2, P1, P2, and P3 axons (e.g., the A/P fascicle in the anterior direction as shown in Fig. 4D). For example, another potentially important pair of identified neurons that contribute axons to this bundle are X1 and X2 (Fig. 2), whose axons extend posteriorly in advance of C's growth cone.

Ultrastructural Analysis of the G Growth Cone and Its Filopodia

We have examined the ultrastructure of the G growth cone, its filopodia, and its filopodial environment (i.e., those cells within filopodial grasp) in serial and semi-serial sections on the transmission electron microscope (TEM). Reconstructions were made from either HRP-filled neurons (Figs. 4C,D, 5, 6, and 7), or from serial thin sections in which individual axons and growth cones were identified according to their location and morphology (Fig. 4A,B). The G growth cone and its filopodia were examined just before it climbed onto the A/P fascicle, or shortly after it began to extend anteriorly upon the A/P fascicle.

There are several other longitudinal axon bundles within 10 μm of the A/P fascicle (Fig. 5). For example, one of these bundles, the D fascicle, runs about 5–10 μm dorsal and slightly medial to the A/P fascicle (shown in Fig. 5) and consists of two axons (the D neurons) just before G extends onto the A/P fascicle. The filopodia of the G growth cone extend for over 30 μm and are within grasp of the D fascicle, the A/P fascicle, and many other axon bundles. Thus, it is of interest to examine the extent of filopodial contact with these different axon fascicles before, during, and after G's growth cone makes its cell-specific choice to extend anteriorly upon the A/P fascicle.

If, as according to Bray's model (1982), filopodia are actively extended and retracted over a several-minute
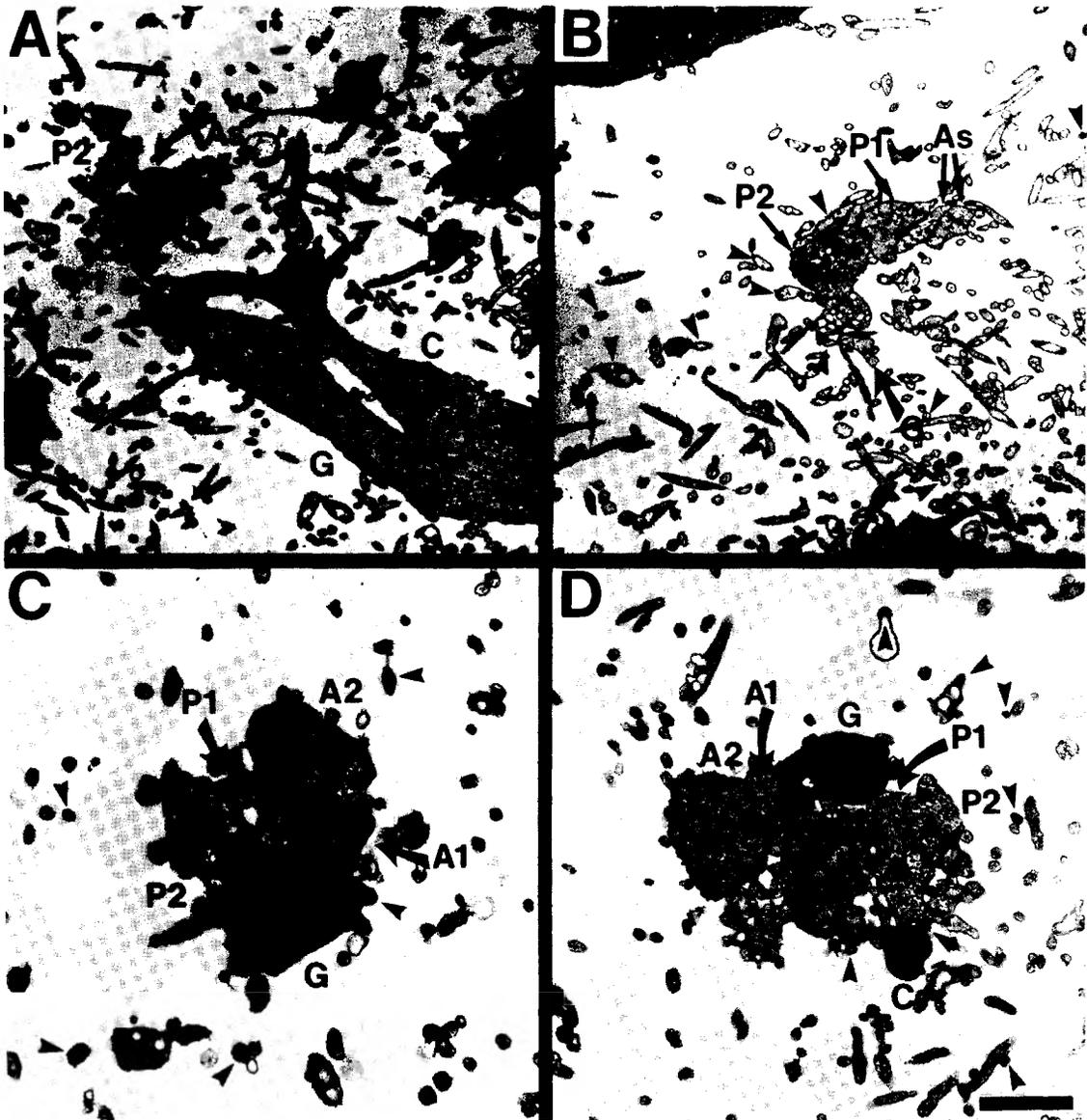
Figure 4. Ultrastructure of the G and C growth cones and their filopodia in relation to the A/P fascicle. Transmission electron micrographs taken from serial thin sections (*A,B*) or semiserial sections of HRP-filled neurons (*C,D*). (*A,B*) Two sections showing the growth cones (*A*) and terminal lamellipodia (*B*) of the G and C growth cones just before the G growth cone gets onto the A/P fascicle. Note the A/P fascicle contains the axons of the A1, A2, P1, and P2 neurons at this time. The G and C growth cones at this stage are suspended, most likely by their filopodia, just medial and ventral to the A/P fascicle. The small black arrowheads in *B* are G's filopodia, and the large black arrow is G's lamellipodium, all of which appear to prefer and wrap around the P axons. (*C*) Electron micrograph of an older stage than that shown in *A* and *B*, showing the A/P fascicle with the A1, A2, P1, and P2 axons, and the G growth cone. Note that the G growth cone is in contact with the P2 axon. Arrowheads show G's filopodia. (*D*) Electron micrograph of an even older stage than that shown in *C*, showing the A/P fascicle with several more axons in addition to A1, A2, P1, P2, G, and C. Bar, *A,B*, 1.5 μm; *C,D*, 1 μm. (*A,B:* Reprinted, with permission, from Bastiani et al. 1984. *C,D:* Reprinted, with permission, from Raper et al. 1983b.)

cycle, then at any one moment, only a small percentage of filopodia will be at their maximum length. However, those filopodia that contact a particularly adhesive surface will retain their full length as their contractile cycle produces tension rather than retraction. Thus, at any one moment, one might expect to find more filopodia contacting the surfaces of particularly adhesive axons, those filopodia being on the average longer than the filopodia touching other axons, and those filopodia run-

ning along and in contact with the adhesive axons for greater distances than the filopodia touching other axons.

In one example from a TEM reconstruction at a time before the G and C growth cones extend onto the A/P fascicle, the G and C filopodia were in extensive contact with all four axons in the A/P fascicle, whereas they made only brief contact with the D fascicle (Fig. 6A). Moreover, they ran along and contacted the axons
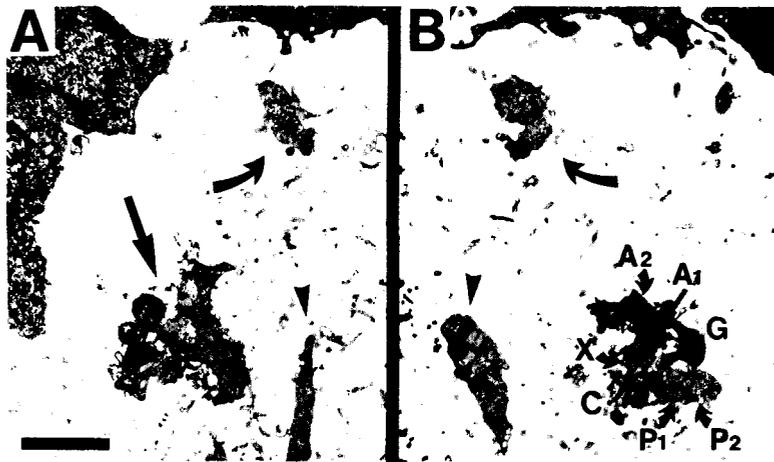
Figure 5. Electron micrographs of two longitudinal axon bundles in the T2 segment of a 41% grasshopper embryo, showing the symmetry of the A/P fascicle and D fascicle on the left (*A*) and right (*B*) sides of the segment. The A/P fascicle (long straight arrow) contains the axons of the A1, A2, P1, P2, G, C, X, and at least two unidentified neurons. The D fascicle (curved arrow) contains the axons of the D1, D2, and D3 neurons. Note a single medial growth cone extending dorsally on both sides (arrowhead). Bar, 5 μm.
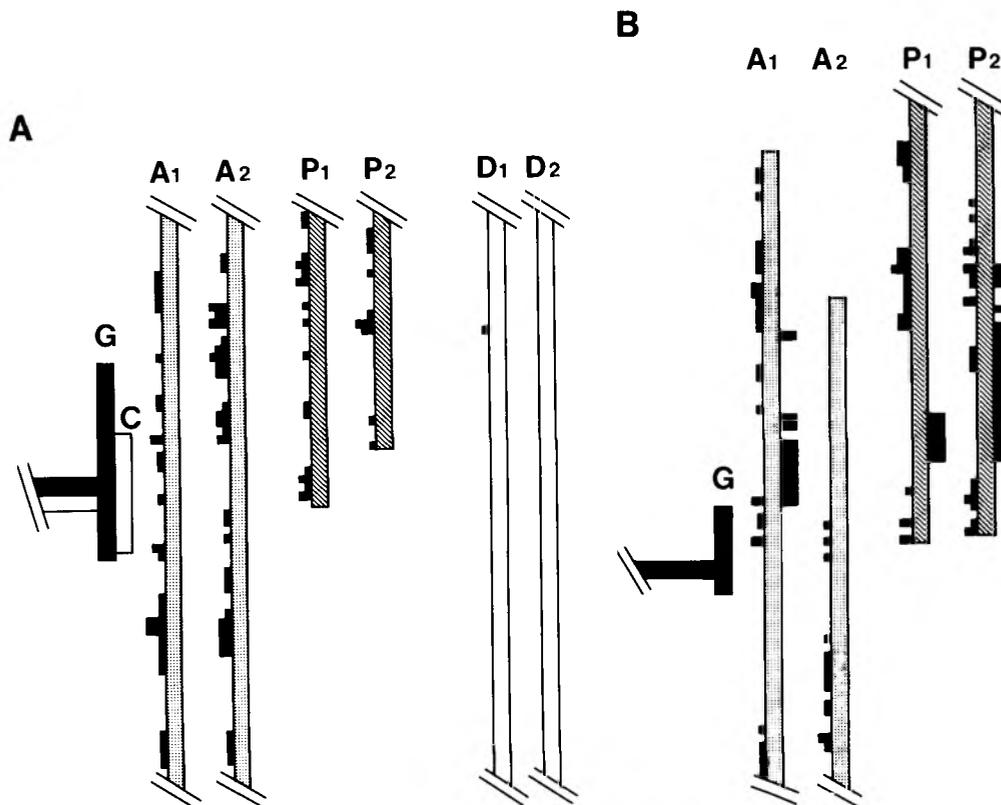
Figure 6. Schematic diagram of the G and C growth cones, their filopodia, and the axons of the A/P fascicle and D fascicle as taken from semiserial TEM reconstructions of HRP-filled neurons, sampled at 1-μm intervals. See text for discussion of results. (*A*) The G and C growth cones have not yet climbed onto the A/P fascicle (A1, A2, P1, and P2 axons). The black bars to the left of the axon profiles represent the extent of filopodial contact with the identified axons. The smallest vertical distance represents 1 μm of contact by one filopodium; the smallest horizontal distance represents one filopodium. (*B*) The G growth cone has just climbed onto the A/P fascicle. The thick black bars to the right of the axon profiles represent the extent of contact by the G growth cone with the identified axons. Note that the tip of the G growth cone is in contact with the P axons. Bar, 25 μm. (Modified from Bastiani et al. 1984.)

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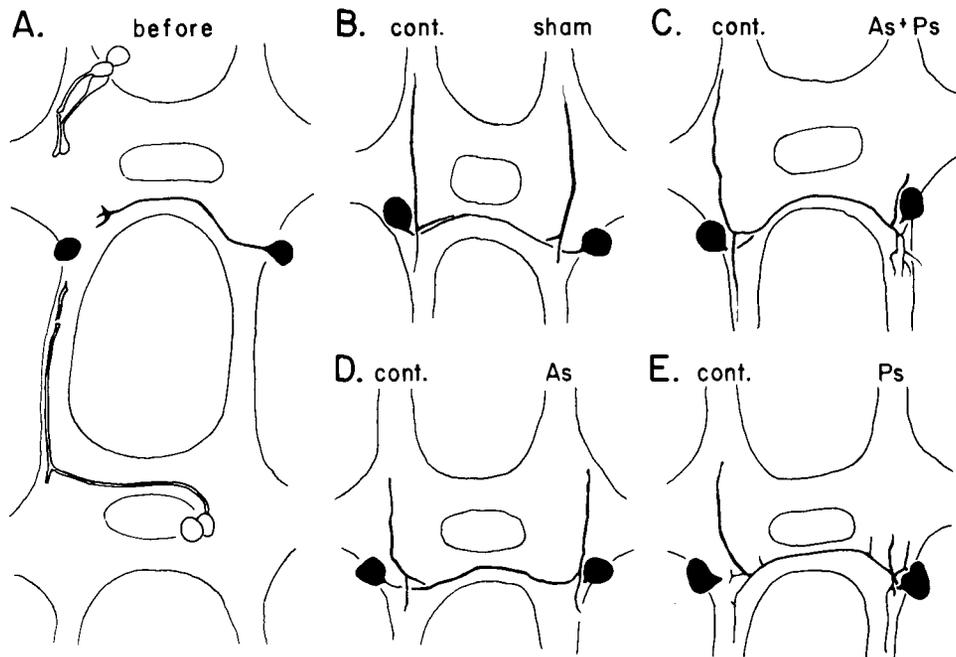
Figure 7. Effects of axon ablations upon the behavior of the G growth cone. The morphology of the G growth cone in examples of manipulated embryos subsequently cultured for 40–48 hr. All manipulations were performed on the right sides, leaving the left sides as internal controls. (*A*) The relative positions of the A1, A2, P1, P2, P3, and G growth cones at the time when manipulations were made. (*B*) Sham manipulated. (*C*) A1, A2, P1, P2, and P3 axons ablated. (*D*) A1 and A2 axons ablated. (*E*) P1, P2, and P3 axons ablated. Bar, 100 μm.

of the A/P fascicle for many micrometers. This suggests that the filopodia of the G and C growth cones more strongly adhere to the surfaces of the axons in the A/P fascicle than to other nearby fascicles.

Before the G and C growth cones extend onto the A/P fascicle, they leave the commissural fascicle containing their sibling's axons and extend dorsally and laterally toward the A/P fascicle (see Fig. 4A). The leading filopodia and lamellipodia from G's growth cone appear to wrap around and prefer the P axons over the A axons at this time (Fig. 4B).

Once G's growth cone has turned anteriorly upon the A/P fascicle, its filopodia continue to be in extensive contact with the A and P axons. Interestingly, the tip of G's growth cone was found in all four cases examined to be closely associated with the P and not the A axons (Fig. 6B). These findings suggest that G is able to distinguish the A/P fascicle from other axon bundles. Moreover, it is likely that it can distinguish the P axons from the A axons. Although the electron micrographs present a static picture of a dynamic process, the results suggest that this selective fasciculation is likely to be mediated by differential adhesion of the filopodia of the G growth cone to the A/P fascicle and, in particular, to the P axons.

Effects of Axon Ablations
upon the Behavior of the G Growth Cone

We examined the effects of ablating the A1, A2, P1, P2, and P3 axons upon the behavior of the G growth

cone. If the A/P fascicle guides G's growth cone anteriorly through 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. For these experiments, the embryos were cultured outside of their eggshell, embryonic membranes, and yolk in RPMI 1640 (GIBCO) culture medium supplemented with sodium bicarbonate, glutamine, sodium pyruvate, glucose, penicillin, streptomycin, horse serum, glycine, bovine insulin, β-ecdysterone, and juvenile hormone I. The ablation of neuronal cell bodies and axons was accomplished with a sharp microelectrode. Two conditioning embryos were added to the culture media and were replaced daily for as long as the manipulated embryo was cultured. Each manipulated embryo had its own internal control, since one G neuron faced a sham or perturbed environment while the contralateral G neuron in the same segment faced a control environment.

The experimental manipulations were performed during a relatively narrow time window (1) after the growth cones of both the A1 and A2 neurons had turned into the ganglionic connectives and (2) before the growth cones of the G, A1, or P1 neurons reached the location at which G turns anteriorly (approximately 37% of embryogenesis; see Fig. 7A). If the embryo is removed from its egg and cultured at 29°C for 40–48 hours, G's growth cone continues extending to a lateral position in the neuropil and then turns onto the A/P

fascicle and extends in the anterior-posterior axis (Fig. 7B). In favorable cultures, the distalmost tip of the 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 that guide G's growth cone through its choice point and anteriorly through the neuropil of the second thoracic ganglion. G's morphogenesis in culture is not entirely normal. For example, G's primary growth cone turns in an almost exclusively anterior direction in ovo, whereas in culture it often branches and extends both a long neurite anteriorly and a shorter neurite posteriorly (e.g., Fig. 7B).

The axons of the A1, A2, P1, P2, and P3 neurons were prevented from making the A/P fascicle in the second thoracic ganglion (1) by cutting the portion of the ganglion connectives in which the A1 and A2 axons run and (2) by killing the P1, P2, and P3 cell bodies and separating them from their axons. Manipulations were performed on one side of the embryo only, so that the opposite side served as an internal control. The morphologies of a control G and sham-manipulated G are shown in Figure 7B. The axons of both neurons extend well anteriorly through the normal, lateral portion of the neuropil. In Figure 7C is shown a preparation in which the A1, A2, P1, P2, and P3 axons were ablated on one side. The control G extends anteriorly past the anterior commissure, whereas the experimental G extends anteriorly for only a short distance. Both Gs have significant posteriorly directed branches. The multiple posteriorly directed processes of the experimental G appear to wander and branch anomalously.

No effect upon G was detected if only the A1 and A2 axons were prevented from joining the A/P fascicle (Fig. 7D). Ablation of only the P cells does effect G's morphology. In Figure 7E 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, whereas several processes on the experimental side make considerably less anterior progress. The two more medial of these processes are growing in abnormally medial positions.

One source of variability from preparation to preparation comes from the variable stretching of embryos as they are flattened 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. When plotted in this manner, manipulations that have no effect should produce points that 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 with the sham-manipulated sides

is very nearly the same for all preparations (Fig. 8A). 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, A2, P1, P2, and P3 axons clearly has a significant effect on G's ability to extend anteriorly (Fig. 8B). The more developed the control side, the more obvious is the deficit on the experimental side. Evidently, G can proceed only with great difficulty more than a short distance anteriorly in the absence of these axons.

Cutting only the A axons appears to have no significant effect upon G's anterior progress (Fig. 8C). Killing only the P1, P2, and P3 neurons produced fairly variable results, which as a whole, indicate that the absence of the P cells does affect G's anterior progress (Fig. 8D). We were able to verify in three preparations that at least one A axon had traversed the second thoracic neuropil.

DISCUSSION

Our descriptive studies showed that the growth cones of the G neuron and its sibling neurons extend in very close apposition to other specific axons in the developing neuropil (Raper et al. 1983b; Bastiani et al. 1984). These observations led us to propose the "labeled pathways" hypothesis (Goodman et al. 1982; Raper et al. 1983b), as described at the outset of this review. This hypothesis predicts that G's growth cone traverses its very precise route in the developing neuropil by first recognizing and then crawling along (1) the axons of its sibling Q1 and Q2 neurons across the posterior commissure, and subsequently (2) the axons of the A1, A2, P1, P2, and P3 neurons. 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 hoped 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 sometimes (1) halting its lateral extension in the contralateral neuropil and (2) elongating on the anterior-posterior axis. This suggests that this aspect of G's behavior may not be solely dictated by the presence of the A or P axons. However, even though none of the A or P growth cones had traversed the choice point when our manipulations were made, some of their longest filopodia were likely to have. Lucifer-filled cellular debris was sometimes evident in the locations in the neuropil previously occupied by the A and P axons, even after 2 days in culture. It is thus possible that signals left on this debris could influence G's behavior.

Ablating the A1, A2, P1, P2, and P3 axons clearly inhibits G's ability to extend anteriorly in the con-
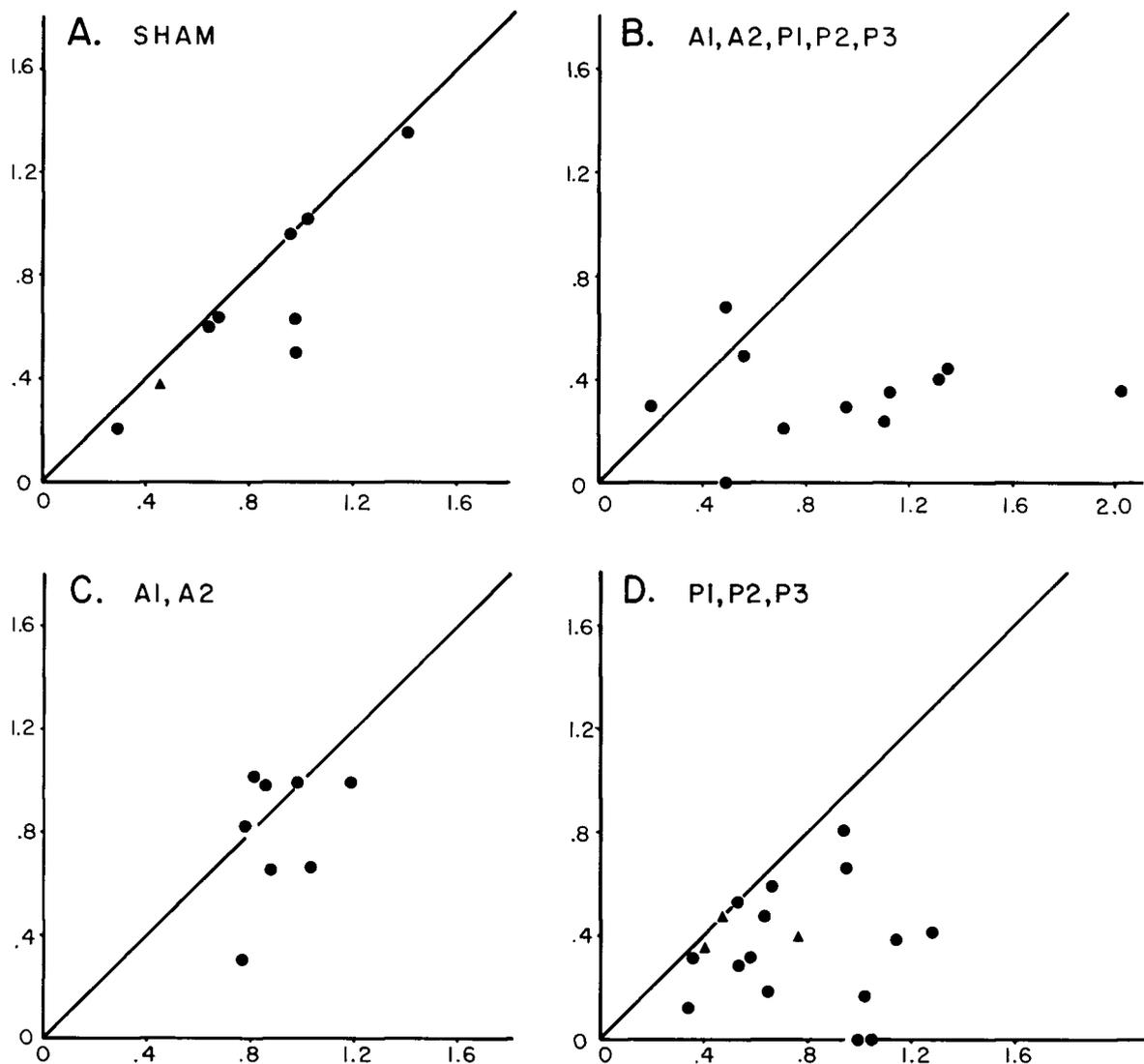
Figure 8. Anterior extension of the G growth cones on the experimental (y axis) as compared with the control (x axis) sides of manipulated and cultured embryos. Lengths expressed as arbitrary dimensionless units (see text). (A) Sham manipulated. (●) Medial portion of ganglionic connective cut and cells adjacent to the P neurons killed; (▲) cells adjacent to the P neurons killed. (B) A1, A2, P1, P2, and P3 axons ablated. (C) A1 and A2 axons ablated. (D) P1, P2, and P3 axons ablated. The three points indicated by triangles represent preparations in which the G growth cones on the experimental side were advancing anteriorly and laterally in anomolous axon tracts. (Modified from Raper et al. 1984.)

tralateral neuropil. This implies that these axons, or a subset of these axons, are required for G's normal anterior elongation. There are several considerations that suggest that the A and P axons play more than a passive role in G's anterior extension. Other axon fascicles less than 10 μm away from the A/P fascicle traverse a parallel route through the ganglionic neuropil around the time when G elongates anteriorly (Bastiani et al. 1984). This distance is small compared with the distance spanned by G's filopodia at the choice point (Raper et al. 1983a).

Experimental evidence points toward an active role in G's guidance 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 or 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. Ablation of only the P1, P2, and P3 axons does. Even when the A axons have traversed the lateral neuropil in the absence of the P axons, G's growth cone does not necessarily 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 A axons. This implies that there are heterogeneous labels within the A/P fascicle itself.

In conclusion, our results show that the A and P axons are required for G's normal extension anteriorly, and they further suggest that the A and P axons are differentially labeled. Although the results from these simple ablation paradigms strongly support the labeled pathways hypothesis, they do not allow us to prove it definitively. At the cellular level, this could best be accomplished by manipulations in which the behavior of G's growth cone is observed after altering the locations of the A and P axons (rather than simply ablating them) in an otherwise intact neuropil.

Thus, it appears that most embryonic growth cones use the surface labels on previously differentiated axonal pathways for selective fasciculation. Can we uncover this postulated code of molecular surface labels? One way is to make monoclonal antibodies that recognize cell-surface molecules specifically expressed on small subsets of axons early in development (Kotrla and Goodman 1983). Such antibodies might potentially reveal the cell-surface molecules used in selective fasciculation. The function of these surface molecules could be tested by applying the antibodies to embryos growing in culture in an attempt to block growth cone guidance (K.J. Kotrla and C.S. Goodman, in prep.). A second way is to take advantage of the recently discovered similarity between the early developing CNS of the *Drosophila* embryo and the grasshopper embryo; cell bodies, axons, and growth cones of identified neurons in the fly embryo appear to be miniature replicas of the same identified neurons in the grasshopper embryo (C.M. Bate et al., in prep.). For example, intracellular dye injections in *Drosophila* embryos reveal neurons homologous to G and C whose growth cones make the same divergent choices in a lateral pathway of the contralateral neuropil. Thus, it should now be possible to uncover the functional role of cell-surface molecules in selective fasciculation by a molecular genetic analysis using *Drosophila* (J. Thomas and C.S. Goodman, in prep.).

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