PATHFINDING BY NEURONAL GROWTH CONES IN GRASSHOPPER EMBRYOS

III. Selective Affinity of the G Growth Cone for the P Cells within the A/P Fascicle[1]

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Abstract

The growth cone of the G neuron selectively fasciculates upon specific axon bundles in a stereotypic sequence as it navigates through the developing central nervous system of the grasshopper embryo. It turns and extends anteriorly in the contralateral neuropil of the second thoracic ganglion at a specific choice point where it fasciculates with the A/P axon bundle which contains the axons of the A1, A2, P1, and P2 neurons. We previously hypothesized (Raper, J. A., M. J. Bastiani, and C. S. Goodman (1983) J. Neurosci. *3:* 20–41) that this fascicle, or subsets of axons within it, were specifically labeled and that the G neuron was determined to follow this labeled pathway.

Here we report on an ultrastructural analysis of the interactions between the G growth cone and its filopodia with the cells of the A/P fascicle at the choice point. As G reaches its choice point, its filopodia are in more frequent contact with the A/P fascicle in comparison to the other longitudinal axon fascicles. Within the A/P fascicle, 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 selective affinity for the P axons as compared to the A axons. Another specific interaction involves selective filopodial insertions; only filopodia from the P cells were found to insert into the G growth cone and induce coated pits and vesicles. 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. The companion paper (Raper, J. A., M. J. Bastiani, and C. S. Goodman (1984) J. Neurosci. *4:* 2329–2345) presents experimental results based on specific axon ablations that further support this hypothesis.

We are interested in the mechanisms that guide neuronal growth cones in the central nervous system (CNS) during embryonic development and have focused our attention on the relatively simple and highly accessible CNS of the grasshopper embryo. We previously described the divergent choices made by the growth cones of the first six progeny of neuroblast (NB) 7-4 and, in particular, examined the morphogenesis of the G neuron (Raper et al., 1983a). The G neuron in the second thoracic ganglion initiates its axon at approximately 35% of embryogenesis. G's growth cone extends within the posterior

commissure across the midline. The G growth cone continues to advance laterally until it reaches a well defined location in the contralateral neuropil. Here its growth cone turns anteriorly, sometimes after a brief pause, and extends many segments toward the brain. G's sibling, the C neuron, follows G to the same location in the contralateral neuropil, but, in contrast to G, it ultimately turns posteriorly and advances into the fused third thoracic and three abdominal ganglia.

We used both dye injection and electron microscopic techniques to examine the location at which the G and C growth cones turn in opposite directions (Raper et al., 1983b). Our hope was to identify some features of the environment which might guide G and C through this critical choice point in the neuropil. One prominent feature of G's environment is the scaffold of nearly orthogonal axon bundles elaborated by previously differentiating neurons. As G 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 which establish this axon bundle, called the A/P fascicle. The axons of the A1 and A2

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cells course anteriorly through the dorsal lateral neuropil. They meet and fasciculate upon the two posterior growing axons of the P1 and P2 neurons to form the A/P fascicle.

These results led us to propose the "labeled pathways" hypothesis for selective fasciculation whereby growth cones are determined to choose amongst specifically labeled axon bundles (Goodman et al., 1982; Raper et al., 1983b). This hypothesis is a new formulation of an idea that has precedent since Cajal's day, and our previous papers discuss its historical development (e.g., Raper et al., 1983a, b, c).

Growth cone motility has been extensively studied *in vitro* (e.g., Bray, 1982; Letourneau, 1982); thus, we thought we might use these *in vitro* studies as a model for our *in vivo* studies. Growth cones radiate many filopodia (approximately 0.1 μm in diameter, up to 50 μm in length) which transiently explore their environment. Many of these filopodia contact other surfaces. To some of these surfaces they strongly adhere, and to others their adhesion is much weaker. If adhesion is weak, during the contractile cycle the filopodium is retracted; however, if 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.

If this model for growth cone guidance from *in vitro* studies (Bray, 1982; Letourneau, 1982) applies to growth cones *in vivo*, then we reasoned that an ultrastructural analysis of the contacts made by growth cones, and in particular by their filopodia, should be indicative of the guidance role and presumably the differential adhesiveness of particular cell surfaces. The labeled pathways hypothesis is based on the notion that filopodia are actively involved in sampling the surfaces of axon bundles within their grasp, and that differential filopodial adhesion mediates their selective fasciculation. The data we present here support this hypothesis by showing that G's filopodia more frequently contact the A/P fascicle as compared to the other longitudinal axon fascicles to which they have equal access. Furthermore, within the A/P fascicle, G's filopodia and the tip of the G growth cone demonstrate a high degree of specificity in their contact of the P axons as compared to the A axons. In addition to selective filopodial contact between the G and P cells, we also demonstrate selective filopodial insertion between these same cells. In the companion paper we present experimental results based on specific axon ablations that further support this hypothesis.

Materials and Methods

Dissections of grasshopper embryos, visualization of the cells with Nomarski optics, and intracellular injections of the cells with either the fluorescent dye Lucifer Yellow (LY) or horseradish peroxidase (HRP) have been described previously (Raper et al., 1983a, b). Single synchronized clusters of eggs were collected from a laboratory colony of *Schistocerca americana*. Dissected embryos were staged first to an approximate age by inspection of their limb buds with a dissecting microscope (Bentley et al., 1979), then to a more accurate age by inspection of the dorsal surface of the neuroepithelium in the second thoracic segment with a Zeiss compound microscope using a Leitz × 50 water immersion lens and Nomarski optics, and finally to the precise age of the G neuron by inspection of the location of its growth cone with fluorescence after filling it with Lucifer Yellow.

Semiserial section reconstructions. The G and C cell bodies can be visualized and individually identified under a compound microscope using a Leitz × 50 water immersion lens and Zeiss Nomarski optics (Raper et al., 1983a, b). Cells at the appropriate stage of development were filled with HRP (Boehringer Mannheim grade I, lyophilized). Embryos were at first dissected out in a hypotonic saline to aid in the visualization (Raper et al., 1983a), but it was later found that the ultrastructure was better preserved when 6 mg/ml of glycine were added to the saline to make it isotonic (Raper et al., 1984). HRP (1%) was dissolved in 0.15 M sodium acetate, Millipore filtered (0.2 μm), and backfilled in glass microelectrodes pulled on a Sutter Instruments microelectrode puller. The final resistance of the electrodes measured

approximately 100 megohms. HRP was iontophoresed into cell bodies by 1.5- to 2.0-nA positive current pulses for several minutes.

After injecting cells with HRP the embryos were fixed for at least 2 hr in a solution of 2% paraformaldehyde and 2% glutaraldehyde in Millonig's buffer, pH 7.2 (Bate, 1976). After fixation the embryos were washed and if necessary stored in cold Millonig's buffer. The embryo was pretreated with a 0.01% $CoCl_2$ solution for 15 min, and then the HRP was visualized by a 3,3-diaminobenzidine and glucose oxidase reaction mixture (Watson and Burrows, 1980). The embryo was then washed in Millonig's buffer, fixed in 2% osmium tetraoxide (2 hr), treated with 1% uranyl acetate solution (1 hr), dehydrated in an ascending ethanol series, and then embedded in Epon/Araldite. After polymerization excess plastic was trimmed from the embryo to allow good visualization of the filled neurons under the compound microscope.

The HRP-filled neurons were drawn and photographed, and appropriate ages were selected for the semiserial section reconstructions. Five different ages and combinations of HRP-filled cells were selected for further study (see Figs. 6 to 10). All of the cells were filled in either the mesothoracic (T2) or metathoracic (T3) segments. The embedded embryos were blocked with a razor blade, glued to a plastic stub, and mounted on a Sorvall MT 2B Ultramicrotome for sectioning. Sections (1 μm) with a glass knife were taken until the boundary of the region to be reconstructed was reached. A diamond knife (Diatome) was then used to section through the entire region. A few sections (silver to pale gold) were collected from each micrometer of sections on Formvar-coated slot grids. Sections were stained with lead citrate and then viewed with a Hitachi HU 11-E electron microscope. Photographs were taken of the best section on each grid.

Sequential analysis of the photographs allowed all of the axons within the field of the sectioned region to be identified. Cells of the A/P fascicle were identified in the reconstructions by their direction of growth, the relative position of their growth cones, and where they got onto and off of the fascicle (Fig. 2). P1 is the first axon extending posterior in the fascicle, P2 is the second, and both P1 and P2 get on the fascicle at the level of the anterior commissure. A1 is the first and A2 the second axon extending anterior in the fascicle, and both remain in the fascicle throughout the segment. X1 and X2 extend across the posterior commissure and get onto the A/P fascicle after G and C. The HRP-filled profiles of G and/or C provided unequivocal identification without the need for reconstruction (Figs. 4B and 5, C and D). Filopodia that were filled with HRP obviously could be identified as originating with the filled cell, but because they could not be reconstructed with a 1-μm sampling interval, filopodia could not be separately assigned to either G or C when both of these cells were filled in the same embryo.

After the cells in the reconstruction were identified, the series of sequential photographs was analyzed for (*i*) the presence or absence of each cell; (*ii*) contact between the HRP-filled profile(s) of the G and/or C growth cones and the other identified cells in the field; and (*iii*) contact between HRP-filled profiles of filopodia and the other identified cells in the field. The schematic reconstructions shown in Figures 6 to 10 summarize the data obtained from each reconstruction. These schematic diagrams provide quantitative information on (*i*) the anterior/posterior extent of the G and C growth cones, and the axons and growth cones of the cells in the A/P fascicle; (*ii*) the degree of contact between the G and/or C growth cones and the cells of the A/P fascicle; and (*iii*) the degree of contact between the filopodia of the G and/or C cells and the cells of the A/P fascicle.

There are two limitations to semiserial section reconstructions of HRP-filled embryonic neurons as described above. The first is that sampling at 1-μm intervals, although more than adequate to reconstruct axons (diameter about 1 to 2 μm), is not useful for reconstructing filopodia (diameter about 0.1 to 0.2 μm). The second is that preservation of the ultrastructure is not optimal in embryos that are dissected out in saline and injected with HRP before fixation.

Serial section reconstruction. An embryo of the appropriate age (see above) was dissected directly into cold (0 to 5°C) fixative. The fixative was 2% glutaraldehyde, 2% paraformaldehyde, 0.2% tannic acid, 2.0 mM $MgCl_2$, and 0.25% dimethylsulfoxide in Millonig's buffer, pH 7.2. After 2 hr the embryo was washed in the above fixative without the tannic acid and transfered directly to 2% osmium tetraoxide in Millonig's buffer for 1 hr. The embryo was further treated with 1% uranyl acetate (1 hr) and 1% tannic acid (1 hr), before dehydrating in an ethanol series and embedding in Epon/Araldite. After polymerization the excess plastic was trimmed away, and the embryo was examined with a compound microscope to confirm the exact stage of development.

The embryo was mounted and sectioned with a glass knife as described above to a position about 10 μm posterior to the G choice point in the T2 segment. A diamond knife was used to cut ultrathin sections from this position to the level of the anterior commissure, a distance of about 60 μm. All sections (about 600) were collected on Formvar-coated slot grids. The grids were stained and viewed as described above, except that a picture was taken of each section on every grid.

The G and C cells and the cells of the A/P fascicle were identified in the 60-μm serial reconstruction as described above for the semiserial reconstructions. All of the filopodia extending from G and C cells, and the cells of the A/P fascicle, were identified from the level of the distal 5 μm of the G growth cone anterior to the end of the longest filopodia extending from the growth cone (about 20 μm). The photograph of each section in this 20-μm region was analyzed for contacts among the identified cells and filopodia (Figs. 12, 13, and 14).

The method of tabulating the contacts for both the semiserial and serial section reconstructions can be seen by examining Figure 11*D*. The *arrowheads* point to three of G's filopodia, and the *arrows* point to six filopodia belonging to the P cells. For each cross-section (the sections used encompassed a much larger field than shown in Fig. 11*D* to include all of the filopodia from the identified cells) the contacts of filopodia and growth cones were tabulated. In this example two of G's filopodia are in contact with the P cells, and one is suspended in space. The G growth cone is in contact with two of the P cell filopodia and two unidentified filopodia. The G growth cone is not in contact with any other axon or growth cone. One P cell filopodium is in contact with a G cell filopodium. The schematic reconstructions shown in Figures 12 and 13 represent a composite account of all of the interactions between the distal G cell growth cone and filopodia and the embryonic environment of the developing neuropil.

Results

The embryonic environment of the developing neuropil in the second thoracic segment at the time and place that the G growth cone makes its choice to grow anteriorly along the A/P fascicle is shown in Figure 1. We were struck by the complexity of axon fascicles at this early embryonic age (39%); yet when both sides of the ganglionic neuropil are compared in a variety of embryos at the same and different ages, it is clear that the spatial and temporal pattern of fasciculation is precise and stereotyped (e.g., Fig. 3). The spaces between axon fascicles are peppered with the 0.1-μm cross-sections of neuronal filopodia extending both from growth cones and as lateral filopodia from axons. We know from our light level analysis of growth cones that these filopodia can extend for up to 50 μm from growth cones, although most are 20 to 30 μm in length (Taghert et al., 1982). A growth cone sitting anywhere within the developing neuropil can sample a large number of different axon fascicles within filopodial grasp. In the course of development the G growth cone passes by about 50 axon fascicles (about 20 longitudinal bundles on each side and about 10 commissural bundles, including those pathways taken by the first two progeny of NB 7-4, Q1 and Q2) to arrive at the point in the contralateral neuropil where G will extend anterior along the A/P fascicle (Fig. 1). At this choice point it can sample up to 25 different axon fascicles but always chooses to extend anterior on the A/P fascicle.

As this location and stage of development, the A/P fascicle contains the axons of four identified neurons: A1, A2, P1, and P2 (Fig. 2). The A/P fascicle forms around 38% of development when the P1 growth cone extending posteriorly along the dorsal basement membrane meets the A1 growth cone extending anteriorly along the same route. Figure 3*B* shows the P1 growth cone at 37% of development extending posteriorly along the basement membrane; several other axon fascicles already exist at this time. When the P1 and A1 growth cones meet around 38%, they fasciculate on one another and "drop" off the dorsal basement membrane to take up their dorsolateral position in the neuropil (Figs. 1 and 4). The A1 and P1 axons do not drop off of the basement membrane in a literal sense. Rather, as they extend along the basement membrane, their filopodia

appear to adhere to the membrane, whereas their axons do not. Once they contact each other, their filopodia appear to have a higher affinity for each other's surface than for the basement membrane. As they then extend along each other's surface, they appear to be pulled taut away from the basement membrane, thus giving the impression of dropping off. Shortly after this stage (39%) the basement membrane is invaded by glial cells that migrate anteriorly, spreading out over its neural surface (Fig. 4). Thus, later growth cones such as G do not have access to the basement membrane initially traversed by the A1 and P1 growth cones.

The rate at which new axon fascicles are formed in the developing neuropil can be seen by comparing Figure 3*A* (37% development) to Figure 1 (39%). The rate at which new axons are added to a pre-existing fascicle can be seen by comparing the A/P fascicle in Figure 1 (39%) to Figure 4 (42%). We have concentrated on an examination of the G and C growth cones and the A/P fascicle during the narrow time window between 39% and 42%. During this period, the A/P fascicle forms and G makes its choice to grow anteriorly along the fascicle (Fig. 5).

The G growth cone, followed shortly thereafter by the C growth cone, extends across the posterior commissure on the Q1 fascicle. When G reaches the lateral region of the contralateral neuropil, it leaves the Q1 fascicle and extends dorsally and laterally to a position just ventral and medial to the A/P fascicle (Fig. 5*A*). The G growth cone remains in this region of neuropil without overtly making a choice to extend anterior for up to 10 hr (2%) (Raper et al., 1983a). Notice that the G growth cone is extending from one pathway, the Q1 fascicle, to another pathway, the A/P fascicle, over a distance of about 20 to 30 μm without a visible substrate in between. Rather, the G and C growth cones seem suspended in space ventral and medial to the A/P fascicle (Fig. 5*A*). Since the G and C growth cones are not growing on a contiguous substrate, this suggests that they must be directed between pathways by extensive filopodial contacts. If we look anteriorly along the A/P fascicle at this time we see that large lamellapodial and filopodial processes from the G cell are contacting cells in the A/P fascicle (Fig. 5*B*). In particular, it appears that the G cell processes are contacting specifically the P cells in the A/P fascicle at this early time (39%). This is especially interesting because after the G cell makes its choice to turn and extend anteriorly (40 to 41%) the tip of the G growth cone is always found on the P cells (e.g., Fig. 5*C*). Whereas the tip of the G growth cone shows a striking preference for the P cells, the axon of the G cell does not. A little later in development (42%) the axon (not the growth cone) of the G cell does not seem to show a strong preference for any particular axons within the A/P fascicle (Fig. 5*D*).

Semiserial reconstructions

These results are shown more convincingly and in more detail in the semiserial and serial reconstructions of the G and C cells and the A/P fascicle. We first present results from the five semiserial reconstructions of HRP-filled neurons. In the first example (Fig. 6), G and C were filled with HRP at a stage before either growth cone had directly contacted the A/P fascicle. A region approximately 70 μm long centered around the G growth cone was sampled at 1-μm intervals. G, C, and the cells of the A/P fascicle were identified (see "Materials and Methods"). In addition, cells of the closest nearby fascicle that extended parallel to the A/P fascicle along the entire region were identified and reconstructed. This pathway, called the D fascicle, forms in the same manner as the A/P fascicle; the D cells start out on the dorsal basement membrane just medial to the Ps and As and then "drop" off the basement membrane to occupy a position just 10 to 15 μm dorsal and medial to the A/
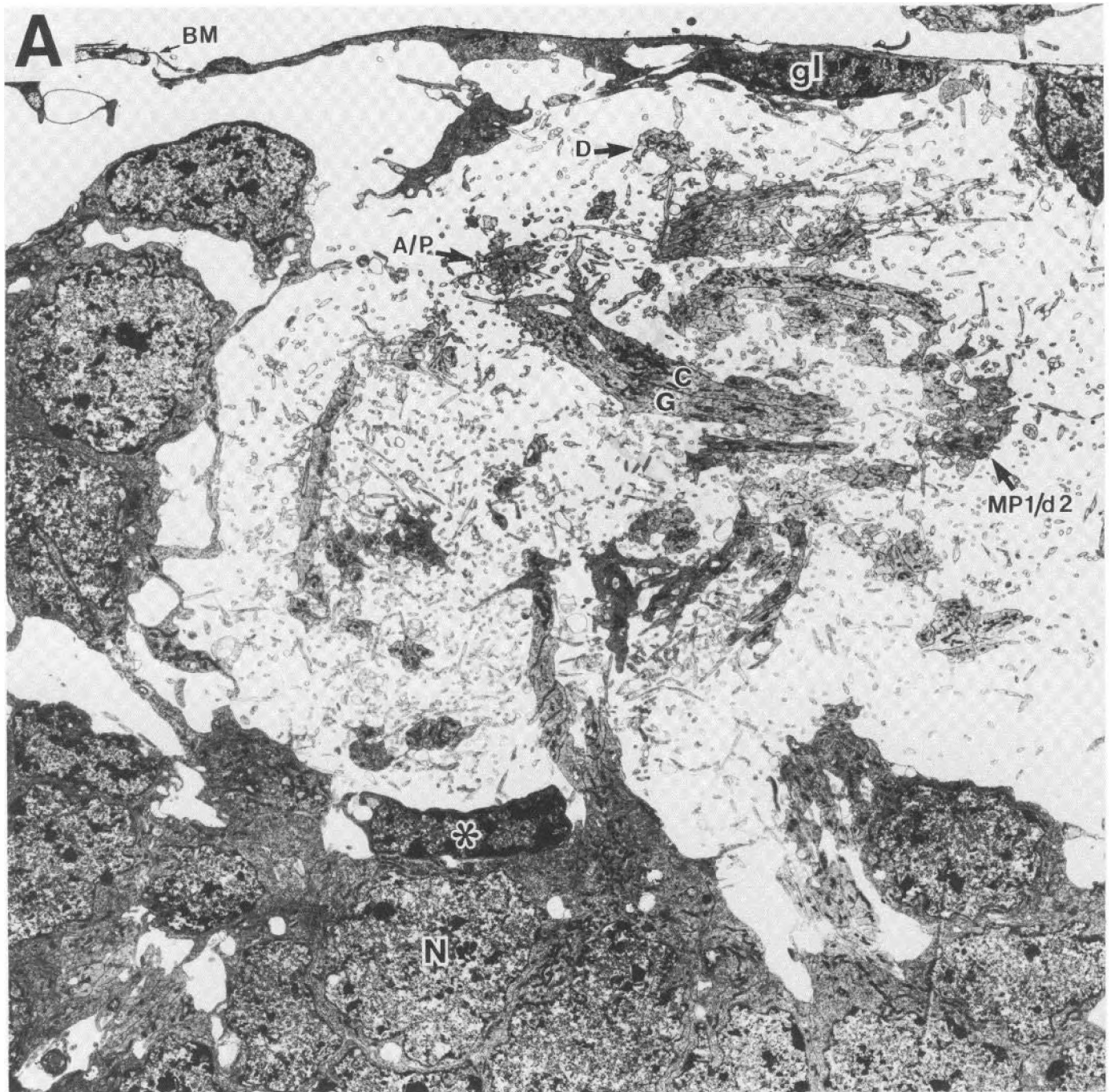
Figure 1. Montage of electron micrographs showing the cellular environment and symmetry of axon bundles at the time G makes its choice to extend anteriorly upon the A/P fascicle in the second thoracic segment (39% of development). *A*, Left side neuropil showing G and C and the A/P fascicle. Notice that virtually the entirety of the contralateral neuropil (about 100 different axons and growth cones in about 25 different axon fascicles) is with 30 μm, and, thus, within filopodial grasp, of the G growth cone. *B*, Right side neuropil showing the symmetry of the axon scaffold. *D*, D fascicle; *MP1/d2*, MP1/dMP2 fascicle; *N*, neuron; *gl*, glia; *BM*, basement membrane. *Calibration bar*: 25 μm. Dorsal is up. *A*, Medial is right; *B*, medial is left.

P fascicle (Fig. 4). The schematic reconstruction of the contacts between the G and C filopodia, and the cells of the A/P and D fascicles, shows that even before the G and C growth cones reach the A/P fascicle their filopodia selectively contact the A/P fascicle in comparison to the other nearby fascicle (D) (Fig. 6). In Figure 6, the *small solid squares* to the left of the axons represent contact between HRP-filled filopodia and the axons of the A/P fascicle (the A1, A2, P1, and P2 cells) and the D fascicle (the D1 and D2 axons).

The next two reconstructions were made at slightly older ages when the G growth cone has just begun to extend anteriorly along the A/P fascicle (Figs. 7 and 8). Only the G cell was filled with HRP in these embryos so that along with the growth cone substrate, the distribution of G's filopodia among the axons of the A/P fascicle could be determined. Both of the schematic reconstructions (Figs. 7 and 8) show essentially the same results; the tip of the G growth cone is found on the P axons and not the A axons. G's filopodia are distributed over the surface
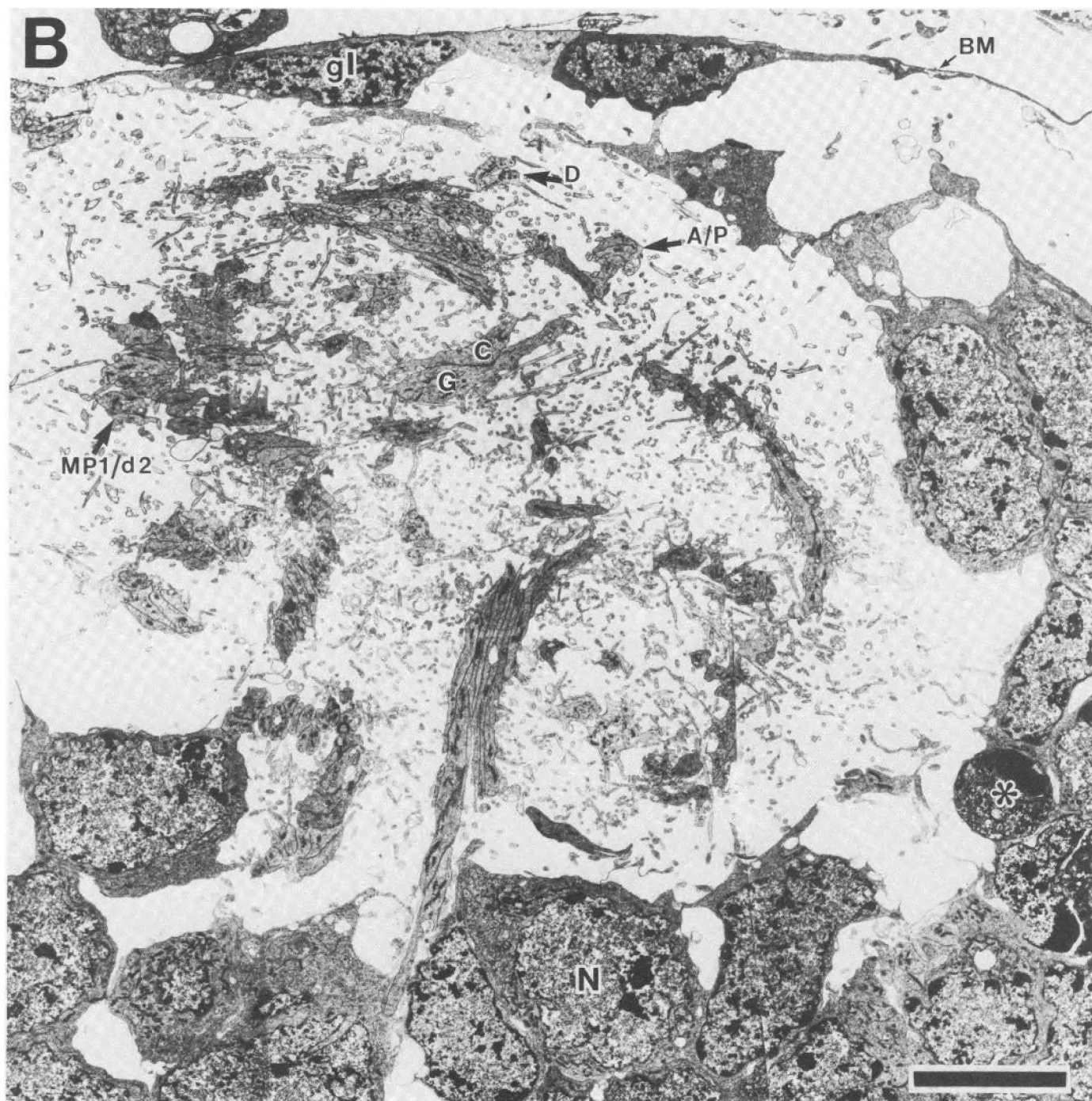
Figure 1B

of both the P and the A axons. As in Figure 6, the *small solid squares* to the left of the axons in Figures 7 and 8 represent filopodial contacts; the *larger solid squares* to the right of the axons represent contact between the G growth cone and the axons of the A/P fascicle.

In Figure 7 the G growth cone is in almost exclusive contact with the P cells. There is only one region of 1 μm where the G growth cone contacts the A1 axon, but notice that even here it is also contacting the P1 axon. The filopodial contacts are spread fairly evenly between the As and the Ps. There is a slight bias in favor of the Ps (52/48%) if the number of contacts for each is simply tabulated and compared. No attempt was

made to normalize for the membrane area or the type of membrane, growth cone versus axon, available to the filopodia. However, when we take into consideration the length of P versus A axon available, the bias in favor of the Ps becomes greater (about 60/40%).

In Figure 8 the G growth cone has extended slightly farther anterior along the A/P fascicle. The tip of the G growth cone is again found on the P axons. There is only a short proximal region of the G cell where it is in exclusive contact with the A1 axon; more distally along the G growth cone, in those few regions where G is in contact with the A1 axon, it is also in contact with the Ps. G's filopodia are again in contact with
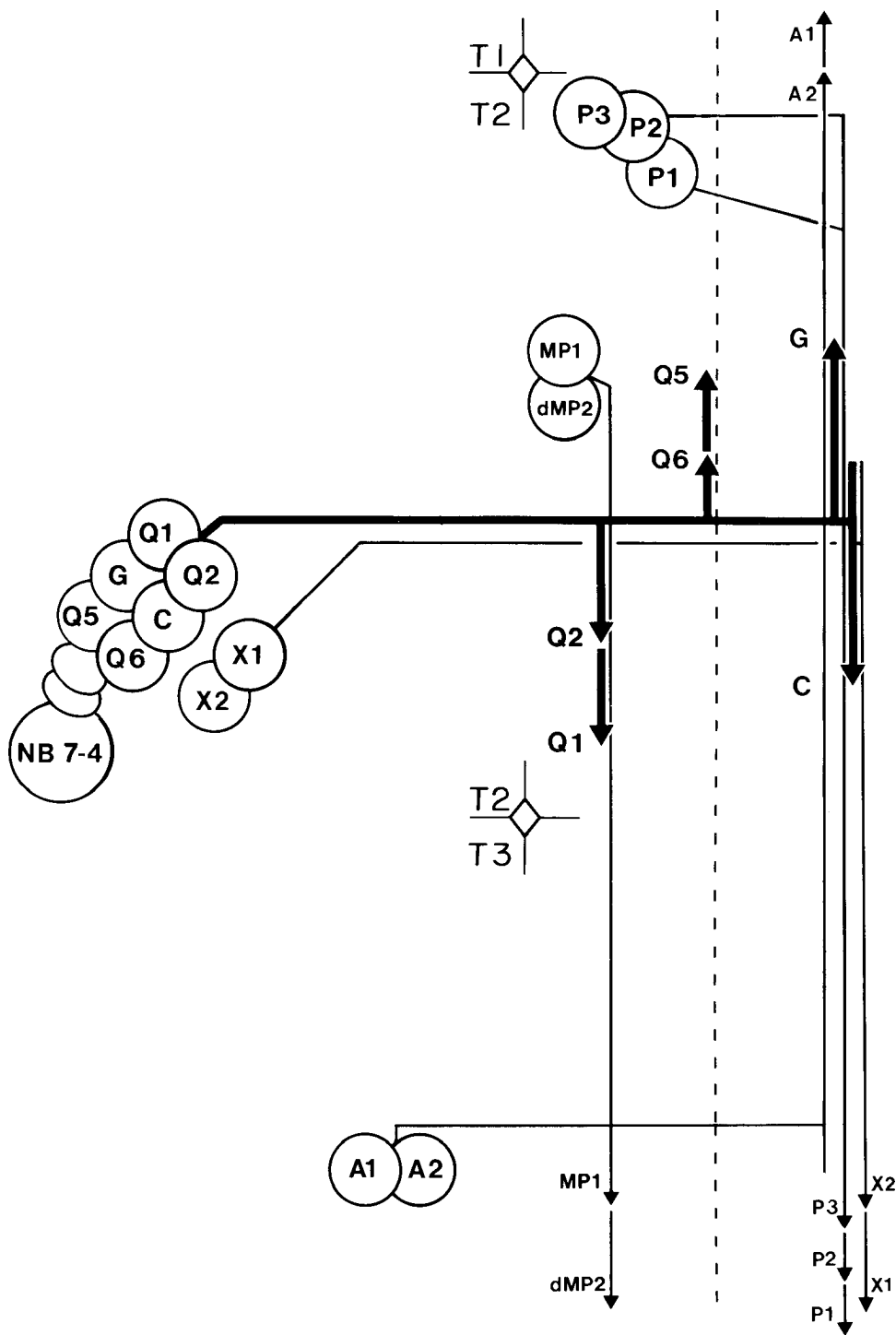
Figure 2. Schematic diagram of the divergent choices made by the growth cones of the first six progeny of NB 7-4. All six growth cones choose the same pathway across the posterior commissure, the Q1 fascicle, yet make divergent choices in the contralateral neuropil. Q1 and Q2 turn posteriorly upon the 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 (*dashed line*). T1/T2 and T2/T3 represent the segmental boundaries. Notice that the Ps are from the same segment as G and C, while the As are from the next posterior segment. The third P cell, P3, is described in the companion paper (Raper et al., 1984).

Figure 3. Electron micrographs of the neuropil in the second thoracic segment at a time (37%) before the A/P fascicle has formed. This is the stage at which the axon ablations are performed in the companion paper (Raper et al., 1984). A, The neuropil region of the entire right half is shown at the level where G and C will make their choice to extend upon the A/P fascicle just a few percentage points later in development (compare this neuropil region at 37% to the same region at 39%, as shown in Fig. 1B). The *arrowheads* point out a few of the early axon fascicles that have already formed. The *asterisk* shows the position along the basement membrane (*bm*) where the As and Ps will meet and fasciculate to
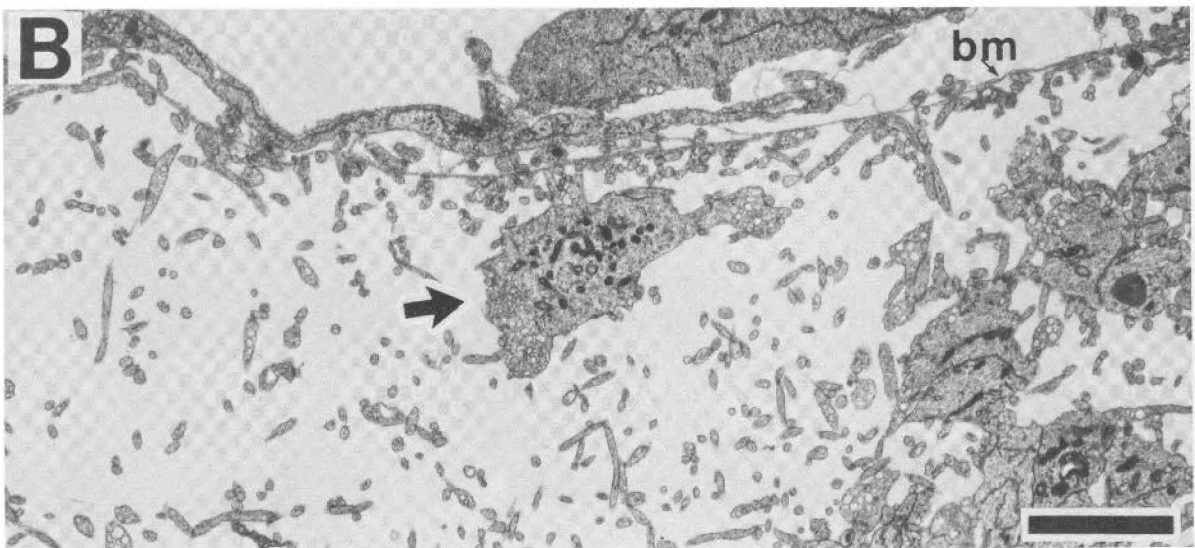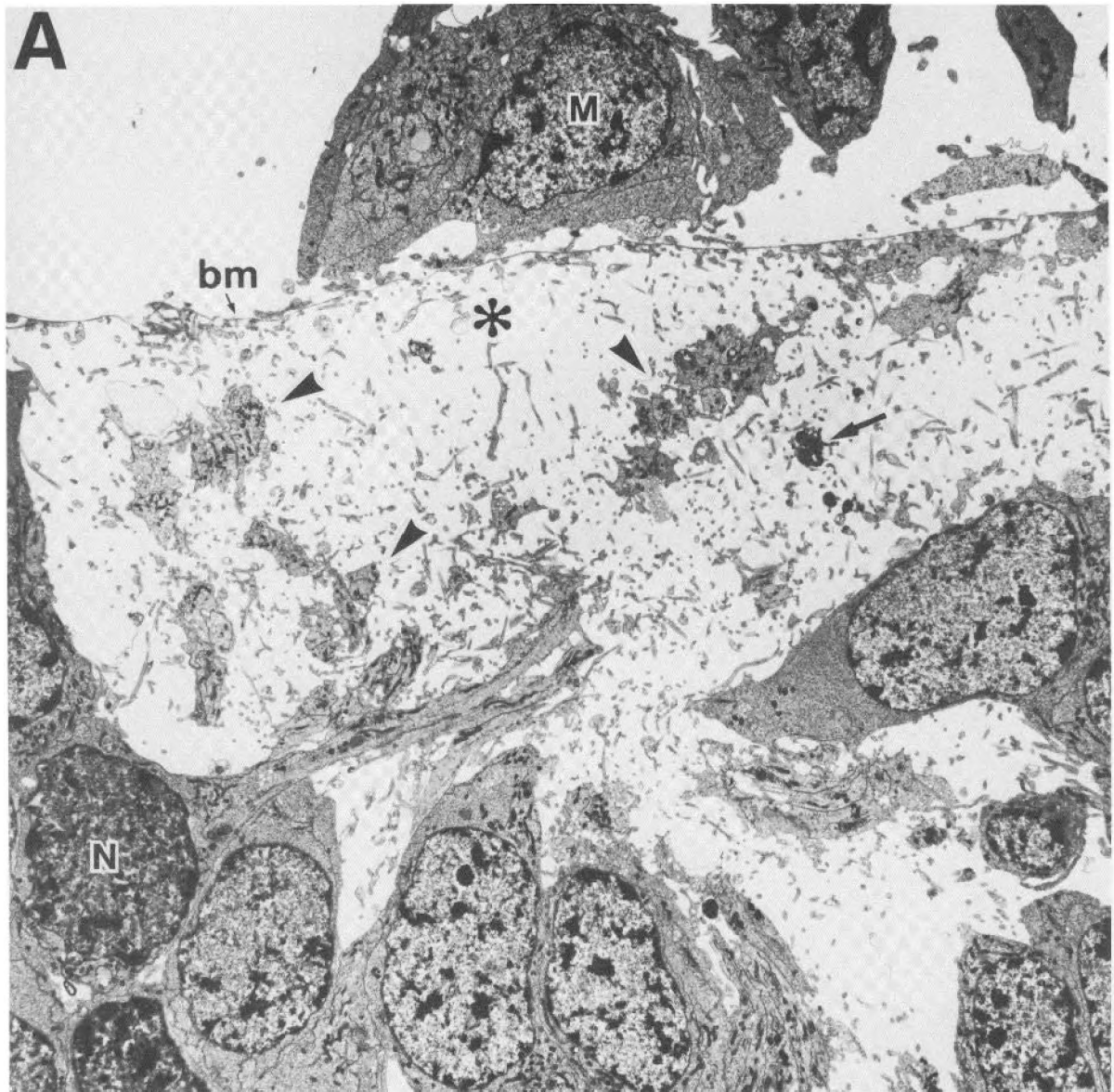
form the A/P fascicle. The *arrow* points out a dying axon, *N* is one of the many neuronal cell bodies ventral to the neuropil, and *M* is a mesodermal cell dorsal to the bm. *B*, A section 60 μm anterior to that shown in *A* but in the same region as the *asterisk* in *A*. The P1 growth cone (*arrow*) is extending posteriorly along the dorsal bm. Within about 1%, the P1 growth cone meets the anteriorly extending A1 growth cone, and they form the A/P fascicle. After they fasciculate, their axons "drop" off the bm to take up the position shown in Figure 1. *Calibration bar:* *A*, 10 μm; *B*, 2 μm. Dorsal is up, and medial is left.
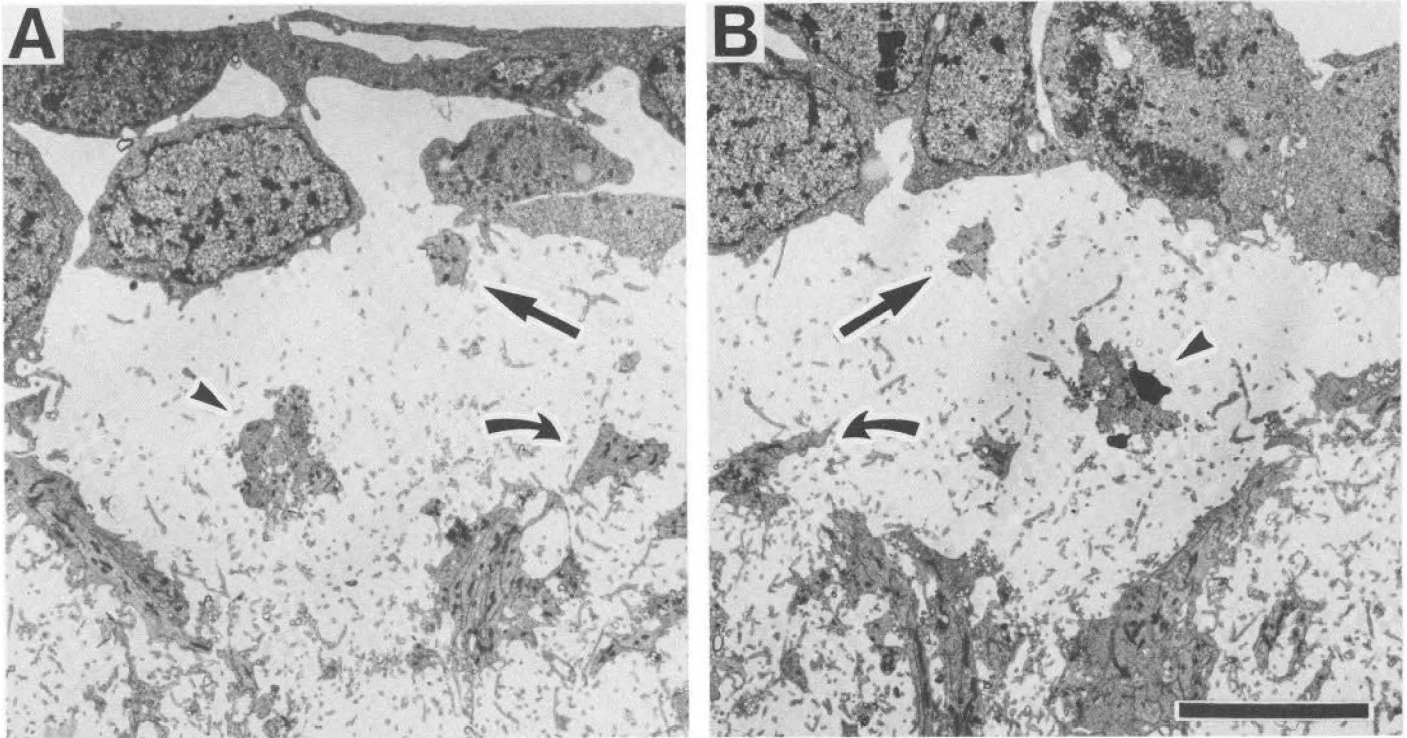
Figure 4. Electron micrographs of three axons bundles in the second thoracic (T2) segment of a 41% grasshopper embryo, showing the symmetry of the A/P fascicle (*arrowhead*), D fascicle (*straight arrow*), and an unnamed third fascicle (*curved arrow*) on the left (*A*) and right (*B*) sides of the segment. The A/P fascicle contains the axons of the A1, A2, P1, P2, G, C, and two axons which are probably P3 and X1 (see Fig. 5D). *Calibration bar:* 10 μm.

both the As and the Ps, and similarly show a slight preference for the Ps (54/46%; again about 60/40% when length of available axon is considered).

In the final two semiserial reconstructions, both G and C were filled with HRP. In Figure 9, G has extended along the A/P fascicle to the level of the anterior commissure while C has grown along G to finally contact the A/P fascicle. The G growth cone is in contact with only the P cells of the A/P fascicle for over 35 μm. The C growth cone is also in contact with the P cells, but this probably does not indicate a preference of the C growth cone for the Ps. Instead it may simply reflect the preference of the C cell for its sibling G since C follows G over to the A/P fascicle.

This interpretation is supported by the results of the last reconstruction at the oldest age (Fig. 10). This schematic reconstruction is different from the previous ones in several important ways. First, the filopodial contacts are not represented; instead the contacts between the C growth cone and axons of the A/P fascicle are represented to the left by the *open rectangles*. The contacts made by the G axon are represented to the right. At this stage of development (42%) the G growth cone has left the segment and entered the longitudinal connective. The region of reconstruction extends only to the anterior edge of the neuropil and does not include the G growth cone. In contrast to those previous reconstructions of the G growth cone, the axon of the G cell in this reconstruction does not show a dramatic preference for the P cells. Second, other axons have joined the A/P fascicle (see Fig. 5D), one of which, the X cell, has been included in the reconstruction. The tip of the C growth cone seems to show a preference for the X axon. The contact between the X axon and the G cell reflects X's choice and not G's since X is extending on the already present G axon. The reconstruction of P1 ends sooner than the others because the proximal axon turns medially toward its cell body (Raper et al., 1983b).

The results of the five semiserial reconstructions can be summarized as follows. (*i*) The filopodia extending from the G and C growth cones show a preference for the cells of the A/P fascicle as compared to other nearby fascicles (Fig. 6 and other data not shown). (*ii*) G selectively fasciculates with the A/P fascicle (Figs. 7 to 10). (*iii*) The tip of G's growth cone is found in contact with the P axons and not the A axons within the A/P fascicle (Figs. 7 to 9), even though its filopodia at this stage only appear to show a small preference for the P axons. (*iv*) The C growth cone may show a preference for the X axon which joins the A/P fascicle after G and C (Fig. 10). (*v*) The G axon does not show the same level of selectivity for the P axons as is shown by the G growth cone (Fig. 10).

The semiserial reconstructions and their interpretation as presented here have several important limitations. First, the small sample size (five) requires that any interpretation be a cautious one, but our previous light level results (Raper et al., 1983a, b), the results presented here, and the results presented in the subsequent paper (Raper et al., 1984) are all consistent with our interpretation. Second, the fixation is not optimal because of the need to dissect out the embryo in saline and then inject the appropriate cell with HRP before finally fixing. More significantly, the process of filling the cells with HRP seems to reduce the number of filopodia seen at light level. The loss of filopodia is probably through retraction and blebbing in response to the penetration and ionophoresis of HRP with the microelectrode using large depolarizing currents. It is doubtful that all of these filopodia still show up in their normal length and position when examined in the transmission electron micrographs (TEM). Third, because filopodia are 0.1 to 0.2 μm in diameter, the sample interval of 1 μm makes it impossible to follow individual filopodia (see "Materials and Methods"), whether filled with HRP or not. The solution to most of these limitations is a serial section reconstruction of an embryo that has been dissected out directly into fixative.
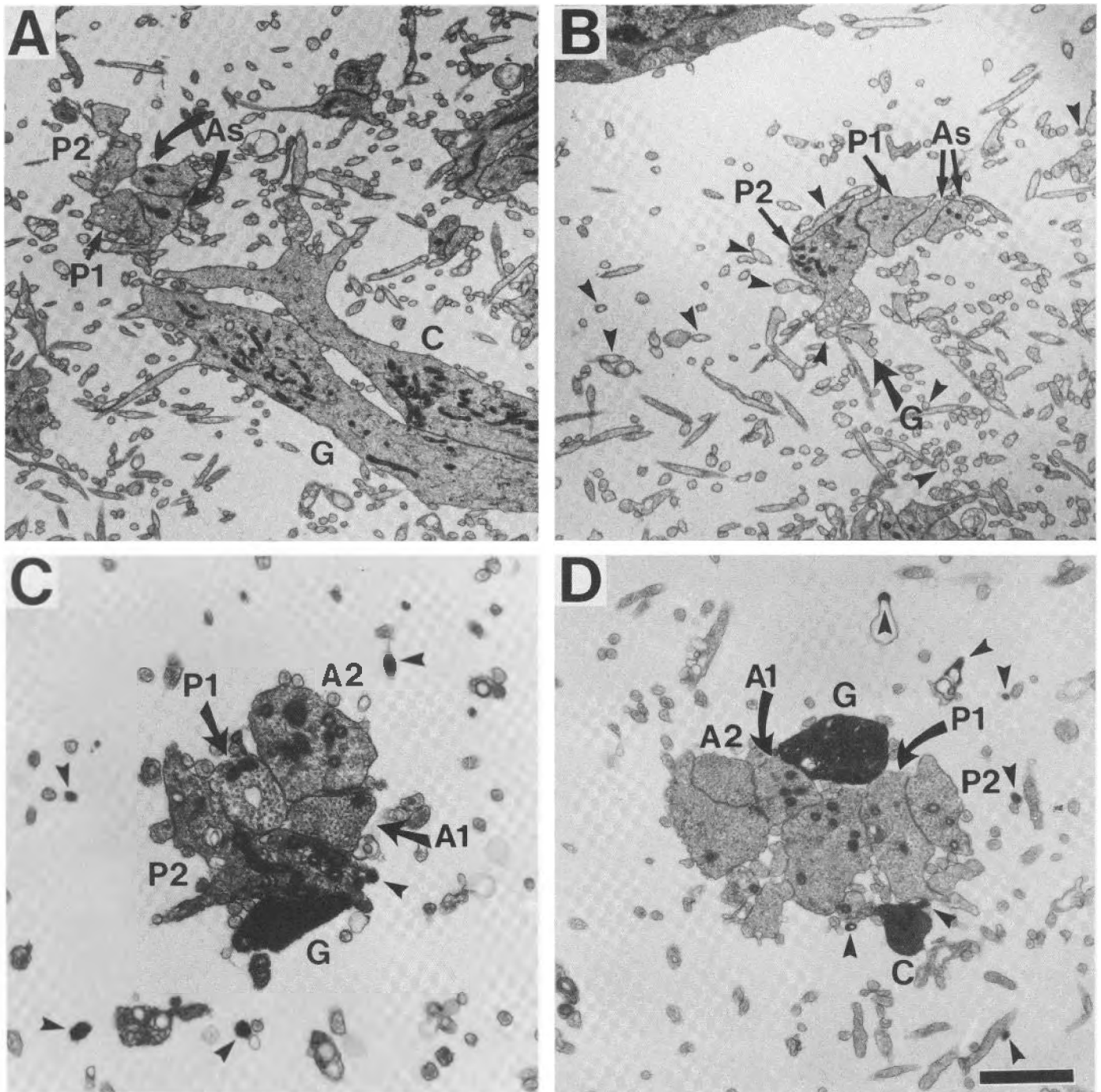
Figure 5. Ultrastructure of the G and C growth cones and their filopodia in relation to the A/P fascicle at three different ages: A and B, 39% C, 40%; D, 41%. Transmission electron micrographs taken from serial thin sections (A and B) or semiserial sections of HRP-filled neurons (C and D). A, G and C are extending across the posterior commissure from the right. Their growth cones are just ventral and medial to the A/P fascicle, which has just formed. Notice that their growth cones are not in direct contact with any other axons; they are suspended by their filopodia. B, Approximately 5 μm anterior to A, the terminal lamellapodia (G) and many of G's filopodia (arrowheads) are seen to be in close contact with the Ps. Very few of G's filopodia contact the As. Notice the presence of growth cone vesicles at the points where the G lamellapodia contact the P growth cone. C, The characteristic close apposition of the tip of the G growth cone with the P axons. The G cell was filled with HRP. The arrowheads point out HRP-filled filopodia from the G cell. D, Both G and C have been filled with HRP. In contrast to the G growth cone, the G axon at this later stage of development (41%) shows no significant preference for the Ps versus the As. Several other axons have already added into the fascicle by this time. A schematic reconstruction of the preparaton shown in A and B is shown in Figures 12, 13, and 14. A reconstruction of the preparation shown in C is shown in Figure 6. A reconstruction of the preparation shown in D is shown in Figure 9. Calibration bar: A and B, 1.5 μm; C and D, 1 μm.

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Figure 7. Schematic semiserial reconstruction showing the contacts of the G growth cone and its filopodia with the cells of the A/P fascicle (A1, A2, P1, and P2). Only G was filled with HRP. Reconstruction was made as described in Figure 6 and under "Materials and Methods." *Bars* to the left of the axons (A1, A2, P1, P2) represent the filopodial contacts as in Figure 6. The *large bars* to the right represent the contact of the G growth cone with the axons. Notice that the G growth cone is in almost exclusive contact with the P1 and P2 axons; the one place where it contacts the A1 axon it is also in contact with the P1 axon. The filopodia at this later time do not show a significant preference for the P axons as compared with the As (compare with Fig. 12). *Calibration bar*: 25 μm.
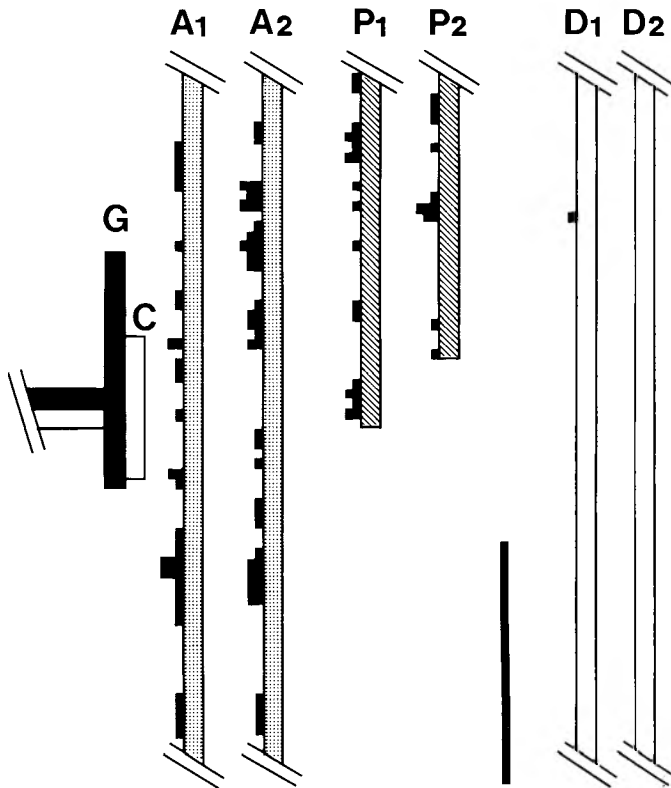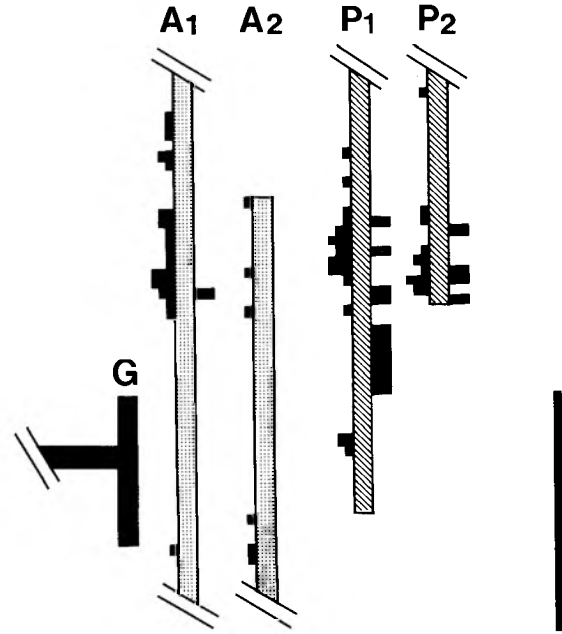
Figure 6. Schematic diagram of the G and C growth cones, their filopodia, and the axons of the A/P fascicle and the D fascicle as taken from a semiserial TEM reconstruction of HRP-filled neurons, sampled at 1-μm intervals. The G and C growth cones have not yet climbed onto the A/P fascicle (A1, A2, P1, and P2 axons). The *solid bars* to the left of the axons represent the extent of filopodial contact with the identified axons; the *smallest vertical bar* represents 1 μm, and the *smallest horizontal bar* represents the cross-section of one filopodium in contact with an axon of the fasicles. Notice that even though the D fasicle is within 10 to 15 μm of the A/P fascicle (see Fig. 4) for the entire distance of its pathway through the neuropil, only one filopodium from the G or C growth cones contacts the D fascicle. This shows that G and C filopodia selectively contact the A/P fascicle before the G or C growth cones have reached this pathway. See also Figure 13. *Calibration bar*: 25 μm.

Serial section reconstruction

A 39% grasshopper embryo was dissected out directly into fixative, prepared for TEM, and serial sectioned from the posterior edge of the neuropil to the anterior commissure, a distance of 60 μm requiring 600 serial sections (see "Materials and Methods"). The G and C cells and the cells of the A/P fascicle were identified as before. In a region including from 5 μm proximal to the distal tip of the G growth cone to the end of G's longest filopodium (about 20 μm), all of the filopodia extending from the G growth cone, C growth cone, and cells of the A/P fascicle were identified. In addition to confirming the information gained by the semiserial reconstructions, this allowed the examination of the interactions of individual identified filopodia with growth cones and filopodia. Two unexpected observations of this reconstruction are (*i*) selective filopodial
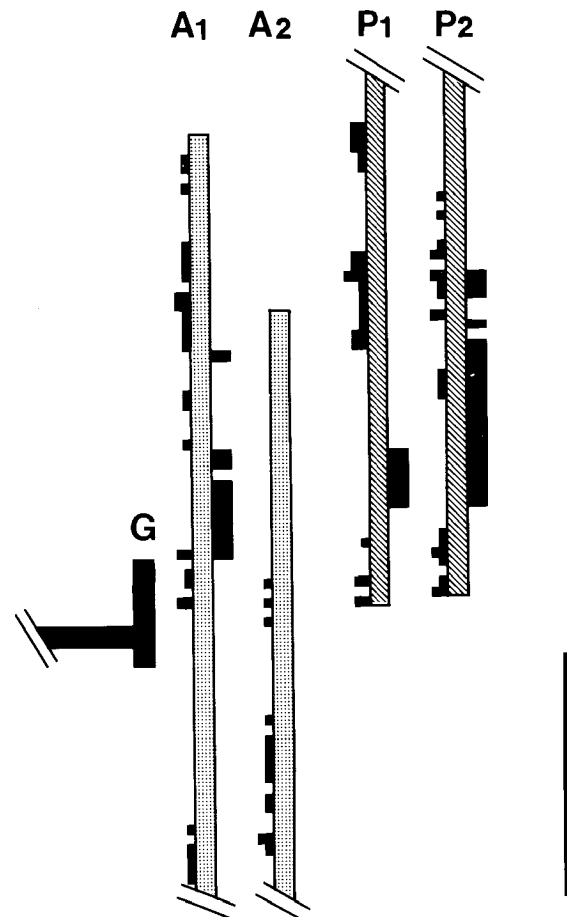

Figure 8. Schematic semiserial reconstruction at a slightly older age than that shown in Figure 7. Only G was filled with HRP. *Bars*, as in the previous two figures, represent filopodial contacts (left) and growth cone contact (right). Notice that, as in Figure 7, the tip of the G growth cone contacts primarily the P1 and P2 cells. Most of the regions where the G cell contacts the A1 cell it is also in contact with the Ps. Filopodia show no significant preference for Ps versus As (see the text and Fig. 12). *Calibration bar*: 25 μm.

insertion (Bastiani and Goodman, 1984a): only filopodia from a P cell insert into the G growth cone and induce coated pits and vesicles; and (*ii*) selective filopodial contact: G's filopodia show a significant bias in their contact with the P cells as compared to the A cells.

Selective filopodial insertion. A series of photographs following the most interesting filopodium is shown in Figure 11, *A* to *H*. The phenomenon of selective filopodial insertion and the specific induction of coated vesicles had been described previously for other identified growth cones in the grasshopper embryo (Bastiani and Goodman, 1984b); thus, it was interesting when we observed the same phenomenon, showing the same degree of specificity, between the P and G cells. The large arrow in Figure 11, *B* to *H*, points out a single P filopodium that penetrates into the G growth cone (Fig. 11, *A* to *D*) and induces coated pits (Fig. 11*A*). Moreover, this same filopodium also
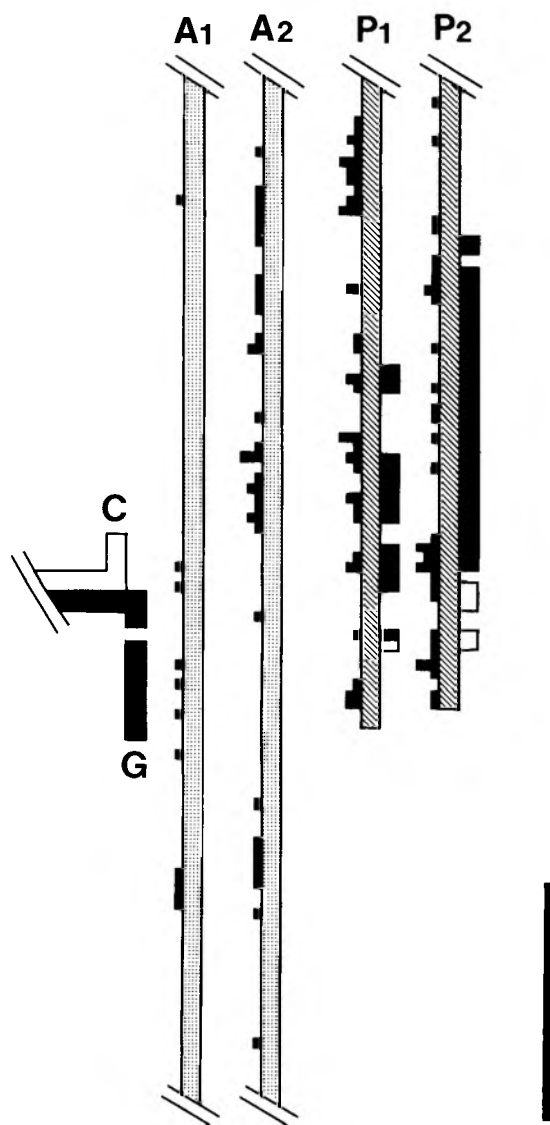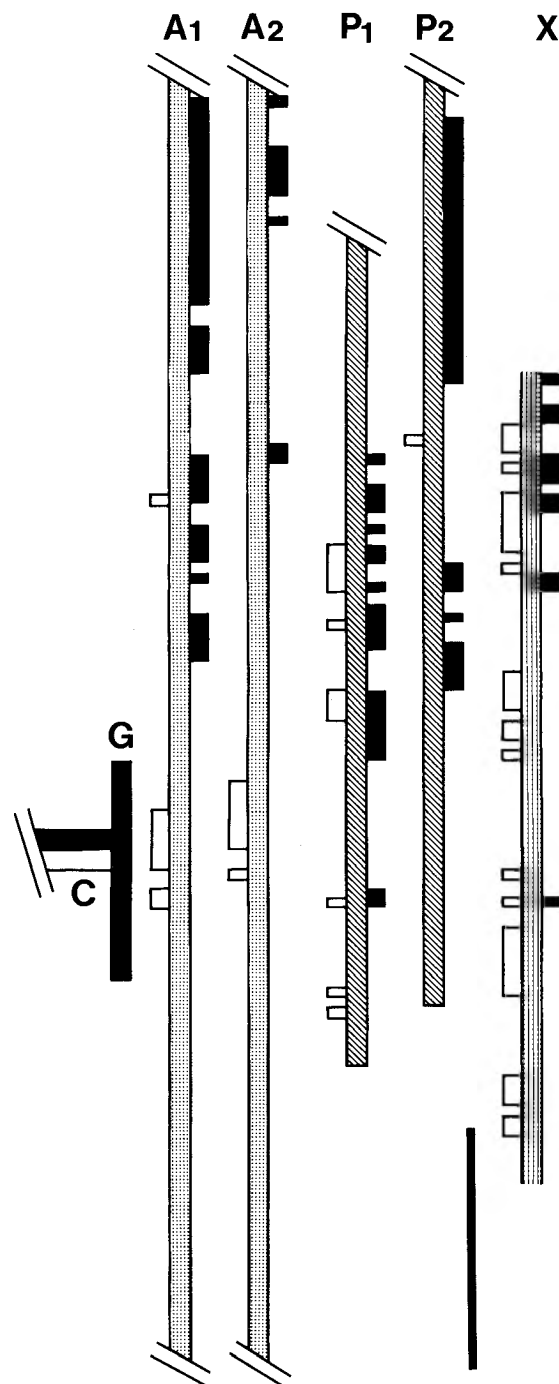

Figure 9. Schematic semiserial reconstruction at a slightly older age than that shown in Figure 8. Both G and C were filled with HRP. *Bars,* as in the previous figures, represent filopodial contacts (*left*) and growth cone contact (*right*). The G growth cone shows a significant preference for contact with the Ps. At this age the C cell has just followed the G cell to the A/P fascicle. It probably contacts the Ps because it follows the G cell that prefers the Ps. C does not appear to make its choice to grow posterior until the X cell joins the A/P fascicle. The filopodia from G and C show a slight preference for the Ps. *Calibration bar:* 25 μm.

Figure 10. Schematic semiserial reconstruction at an age older than that shown in Figure 9. *Bars* to the *left* represent the contacts of the C growth cone, and *bars* to the *right* represent the contacts of the G growth cone. The filopodial contacts are not shown. At this age the G growth cone has left the segment and entered the intersegmental connective; the reconstruction ends at the anterior border of the segment and does not include the G growth cone. Notice that the G axon does not show a preference for the Ps versus the As. The C growth cone appears to contact the X axon selectively. *Calibration bar:* 25 μm.

appears to be the substrate on which the G growth cone is extending to the P cell (Fig. 11, *A* to *H*).

Figure 11*A* is 0.1 μm distal to the tip of the P filopodium. A coated pit region (*arrow*) and dense-core membraneous sacs (*arrowhead*) that also seem to be associated with the endocytotic events at the tip are clearly evident. The densely staining tip of the filopodium in the sleeve of the G cell is pointed out by the *arrow* in Figure 11*B*. With the exception of the tip, the
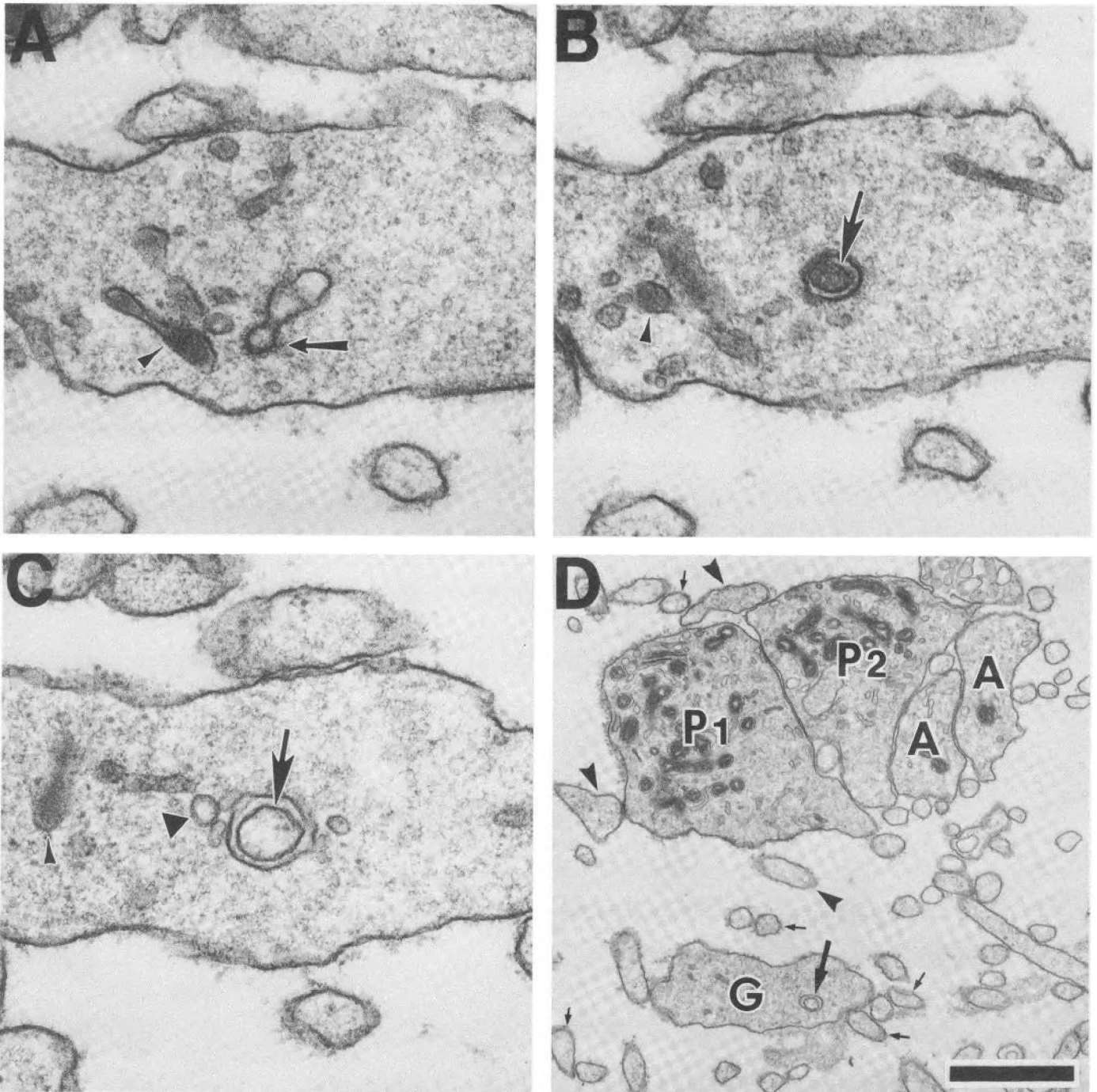
Figure 11. Selected electron micrographs from the serial section reconstruction of the G and C cells and the cells of the A/P fascicle showing the interaction between the G growth cone and filopodia, and the cells and filopodia of the A/P fascicle. The schematic reconstruction of the entire series is shown in Figures 12 to 14, *A* to *H*. Posterior to anterior from the tip of the G growth cone to the tip of G's terminal lamellapodium (approximately 10 μm). *A*, The induction of coated pits (*arrow*) by a P filopodium that has inserted into the G growth cone. Notice the dense-core membranous sacs (*arrowhead*) associated with the tip of the insertion. The cytoplasm of the distal growth cone is generally quite translucent and organelle free. *B*, The section adjacent to that in *A* shows the relatively dense tip of the P filopodium (*arrow*). The *arrowhead* shows the dense membranous sacs associated with the tip of this filopodium. *C*, The inserted P filopodium (*arrow*) now has a translucent cytoplasm. Smooth translucent vesicles (*triangle*) are associated with the sleeve formed by the G growth cone around the P filopodium. The amount of dense-core membranous sacs has dramatically diminished (*arrowhead*). This section is 0.2 μm anterior to *B*. *D*, Micrograph showing the relationships among the G growth cone and its filopodia, the cells of the A/P fascicle, and the P filopodia. Many of the filopodia (*small arrows*) that are in close contact with the G growth cone belong to the P cells. The P filopodium inserted into the G growth cone (*large arrow*) is the same shown in *A* to *C*. The same P filopodium is followed back to the P1 cell (*large arrow* in *D* to *H*). Also notice the large G lamellapodia (*arrowheads*) contacting the P cells. The P1 and P2 profiles are quite large and filled with organelles because this is a section through the main part of the P growth cones, while being proximal to the main part of the A growth cones. *E* to *H*, The P filopodium (*large arrow*) that was inserted into the G growth cone has just left the interior of the G cell and is now running in close apposition to the surface of the growth cone (*E*). Notice the G lamellapodia and filopodia (*arrowheads*) in contact with the P cells and the P cell filopodia (*large* and *small arrows*) in contact with the G growth cone, lamellapodia, and filopodia (*E* to *H*). The G cell terminal growth cone and lamellapodia seem to have followed the inserted P filopodium back to the P1 cell (P filopodium, *large arrow* in *A* to *H*). The profiles of the As and Ps are more nearly the same diameter proximal to the growth cones (*F* to *H*). *Calibration bar*: *A* to *C*, 0.2 μm; *D* to *H*, 2 μm.

cytoplasm of the filopodium is relatively translucent and orga-
nelle-free; similar to the tip of the G growth cone and lamella-
podia (Fig. 11C). Notice in Figure 11C that smooth translucent
vesicles (*triangle*) are associated with the sleeve surrounding
the P filopodium proximal to the tip; these may reflect the
mechanism of sleeve formation and elongation around the P
filopodium. The amount of dense-core vesicular material (*ar-
rowhead*) decreases significantly from Figure 11, A and B to C
and D. The tip of the G growth cone is a few micrometers
ventral to the A/P fascicle and is surrounded by filopodia from
many other sources in addition to the Ps (*arrows*, Fig. 11D).

Selective filopodial contact. Several lamellapodia from G (Fig.
11D, *arrowheads*) can be seen wrapping around the P cells of
the fascicle. The difference in the size and cytoplasmic features

of the Ps and the As (Fig. 11, D to H) reflects growth cone
versus axonal region of the cells in the fascicle: the thicker
processes with many mitochondria are growth cones, whereas
the thinner processes are axons. The semiserial reconstructions
suggest that differences in filopodial contact are not just the
result of differences in growth cone versus axonal membrane
(Figs. 6 to 9). The series of photographs (Fig. 11, D to H) show
that G's lamellapodia and filopodia primarily contact the P
cells and not the As, even though G's processes should have
equal access to both sets of cells. In addition, P's filopodia (Fig.
11, D to H, *arrows*) are the only ones in constant contact with
the G growth cone and terminal lamellapodia. Finally, the P
filopodium that inserts and induces coated vesicles in the G
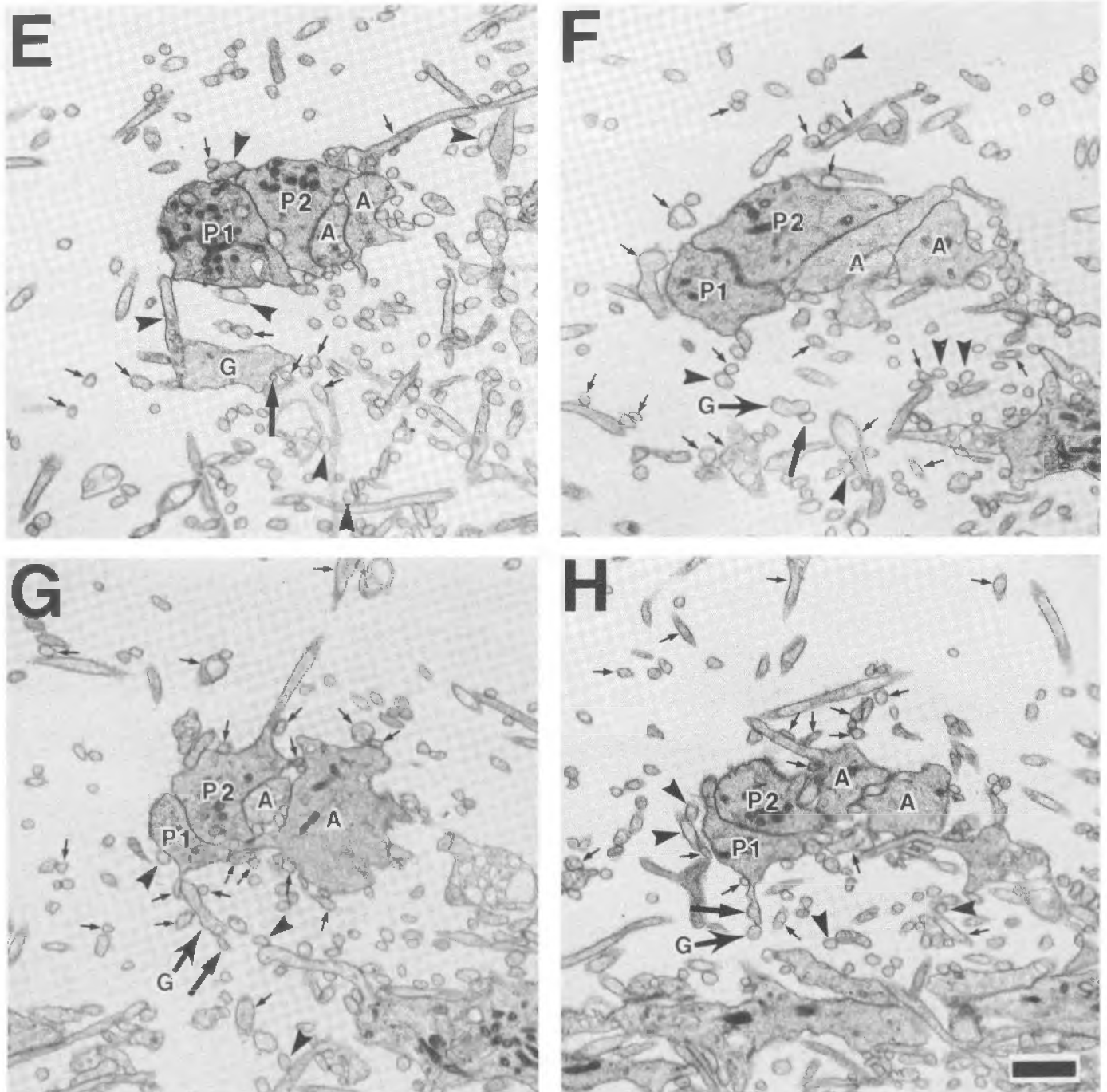growth cone remains in constant contact with the G growth

Figure 11, E to H

cone and terminal lamellapodium; G seems to follow this filo-podium all the way back to its origin at the P1 cell (*large arrow*, Fig. 11, *B* to *H*).

The serial section reconstruction is presented schematically in Figures 12 to 14. Figure 12 represents all of the contacts
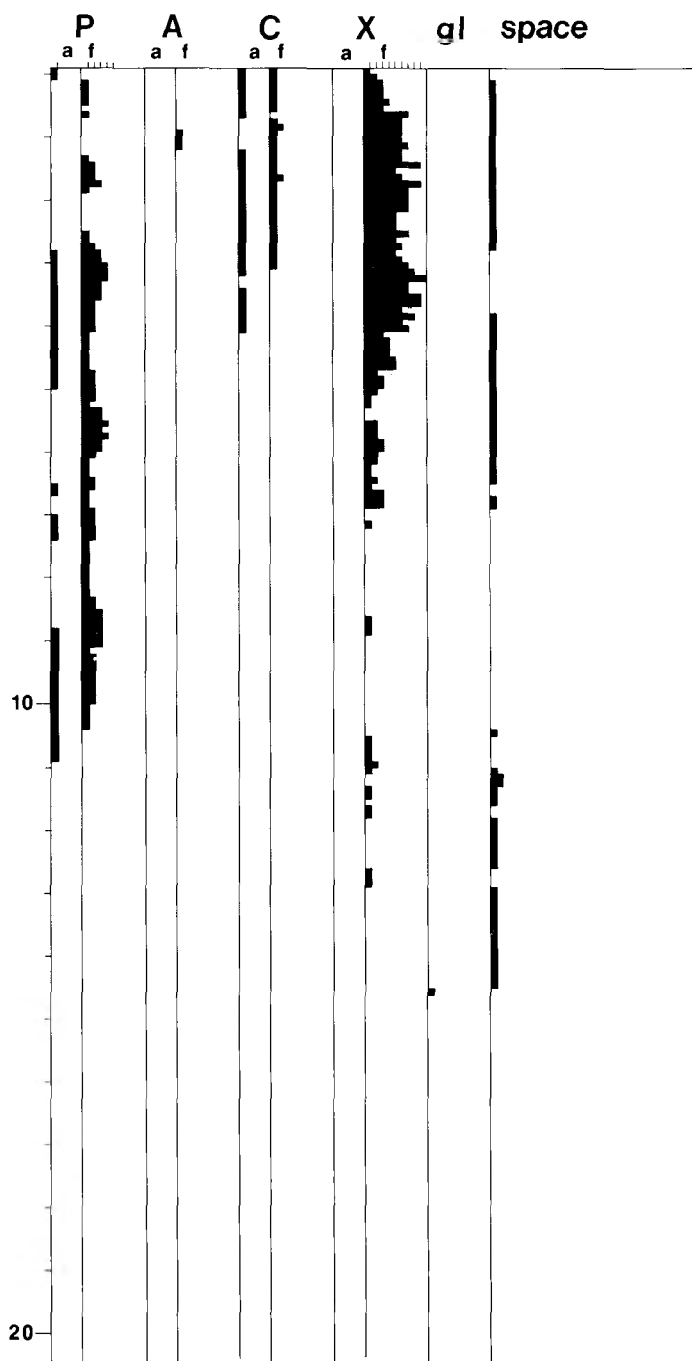

P   A   C   X   gl   space

Figure 12. Contacts by the G growth cone. Schematic representation of the serial section reconstruction of the G and C growth cones and filopodia, and the cells of the filopodia of the A/P fasicicle (examples of the sections are shown in Fig. 11; see also Figs. 13 and 14). The most distal (anterior) 5 μm of the G growth cone were reconstructed along with all of the filopodia that originated from this portion of the growth cone. When the growth cone tapered to less than 0.5 μm it was arbitrarily called the terminal lamellapodium. This schematic shows all of the cells and processes in contact with the G growth cone and terminal lamellapodium. The G cell has not fully grown onto the A/P fascicle at this time and is suspended in space for much of its length ventral to the A/P fascicle. *P* represents both of the P cells; *A* represents both of the A cells. *C* represents the C cell; *X* represents all other axons and growth cones in the region (about 100). *gl*, glia; *space*, regions

made by the G growth cone and terminal lamellapodia. Figure 13 represents all of the contacts made by the G filopodia; Figure 14 represents all of the contacts made by the C filopodia. The six categories in Figure 12 encompass all of the possible envi-ronments of the G cell: the two P cell axons (*Pa*) and their filopodia (*Pf*); the two A cell axons (*Aa*) and their filopodia (*Af*); the C growth cone (*Ca*) and its filopodia (*Cf*); all uniden-tified axons (*Xa*) and their filopodia (*Xf*) (not to be confused with the identified X1 and X2 axons, which are not present in this region at this time); all glial and epidermal cells (*gl*); and, finally, if the G growth cone was not in contact with any other axon except C, then it was put in the *space* category.

The most important comparison is between the G growth cone's contact with the P cell axons and filopodia (*Pa,f*) versus its contact with the A cell axons and filopodia (*Aa,f*). The G growth cone and terminal lamellapodia are in almost continu-ous contact with filopodia from the P cells (*Pf*), while they are virtually never contacted by filopodia from the A cells (*Af*). Lamellapodia extending laterally from the G growth cone con-tact the P axons (Pa) but never the A axons (*Aa*). Contacts with the C growth cone and filopodia (*Ca,f*) reflect C's choice in using its sibling G as a substrate to the A/P fascicle. The G growth cone does not come into contact with any other axons (*Xa*) but is contacted by numerous unidentified filopodia (*Xf*). Although there is a large number in this category it must be remembered that these filopodia probably come from about 100 different growth cones and axons in the approximately 25 different axon fascicles within 30 μm of the G growth cone. We do not feel that there are any hidden specificities important for the guidance of the G growth cone in this category because (*i*) the contacts are generally short, a few tenths of a micrometer; (*ii*) no unidentified axons are contacted by the G lamellapodia; and (*iii*) for much of the length of the terminal lamellapodium (7 to 10 μm), G is in contact almost exclusively with P filopodia. Glial (*gl*) contacts with the G growth cone are very rare and can probably be ruled out as having any importance in the guidance of G to the A/P fascicle. At this time in development (39%) the G cell has not made the choice to grow anterior along the A/P fascicle, as assayed by the growth cone's morphology (Raper et al., 1983b). This is reflected in the length of the G growth cone that is not in contact with any other axon except C (e.g., *space* in Fig. 12).

The second schematic drawing (Fig. 13) represents of all the contacts of G's filopodia with the same categories as in Figure 12. Again, the most interesting comparison is between the number of contacts of G's filopodia with P cell axons and filopodia (*Pa,f*) versus its contact with A cell axons and filo-podia (*Aa,f*). Even though access to the As and the Ps appears to be equal, G's filopodia clearly contact the P axons and filopodia much more than the As. This results shows a more striking bias by the G filopodia for the P axons than the results shown by the two semiserial reconstructions (Figs. 7 and 8) where G was filled with HRP. But there are important differ-

where the G growth cone or terminal lamellapodium were not in contact with any other cell (except C and filopodia). *a,f* represents the axons and filopodia, respectively , of the P, A, C, and X cells. The region of overlap between *space* and *Pa* (from 3.5 to 5 μm) represents a splitting of the G growth cone into two lamellapodia, one of which contacts the P axon and the other of which (the main one) remains suspended in space. The contact with glia is minimal, but the G cell is contacted by numerous unidentified filopodia (*Xf*). The contact with the C cell (*Ca, f*) is indicative of the specificity of the C cell for the G cell rather than the reverse since the C cell is extending on G. The major comparison is between the *Pa,f* and *Aa,f*. Even though G, the Ps, and the As have similar access to one another, there is a dramatic difference between the contact of the P cells (*Pa,f*) versus the A cells (*Aa,f*) with the G cell growth cone and lamellapodia. The *vertical scale* is distance in micrometers, 1 μm/division (10 sections). The *horizontal scale* is the number of filopodial cross-sections, one filopodium per division.
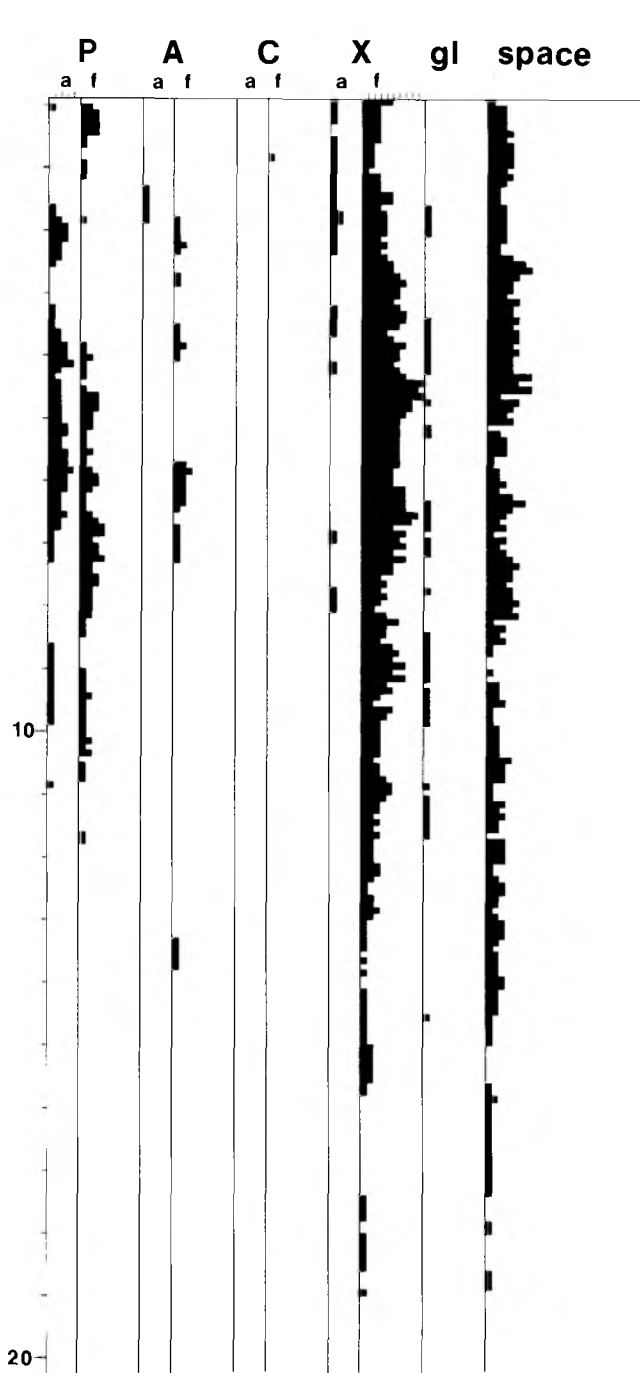
Figure 13. Contacts by the G filopodia. This is a schematic reconstruction of the same cells as in Figure 12. This shows the relationship between the filopodia extending from the anterior 5 µm of the G growth cone and the P cells (*Pa,f*), A cells (*Aa,f*), C cell (*Ca,f*), unnamed cells (*Xa,f*), glial cells (*gl*), and *space*. The G filopodia are not in contact with any cells or other filopodia for a significant portion of their length (*space*). There is only minor contact with glia cells and other unidentified axons (*Xa*), but many with unidentified filopodia (*Xf*). X represents the approximately 100 other axons and growth cones in the neuropil within filopodial grasp of the G growth cone. The small number of contacts with the C growth cone is not surprising given the fact that C is behind and growing upon the G growth cone. Again the major comparison is between the A cells (*Aa,f*) and the P cells (*Pa,f*). The G cell filopodia have equal access to both As and Ps, yet they show a significant preference for contact with the Ps. Compare *Pa* versus *Aa* and *Pf* versus *Af*. As in Figure 12, *a* and *f* refer to the axons and filopodia, respectively. The *vertical* and *horizontal scales* are as in Figure 12.
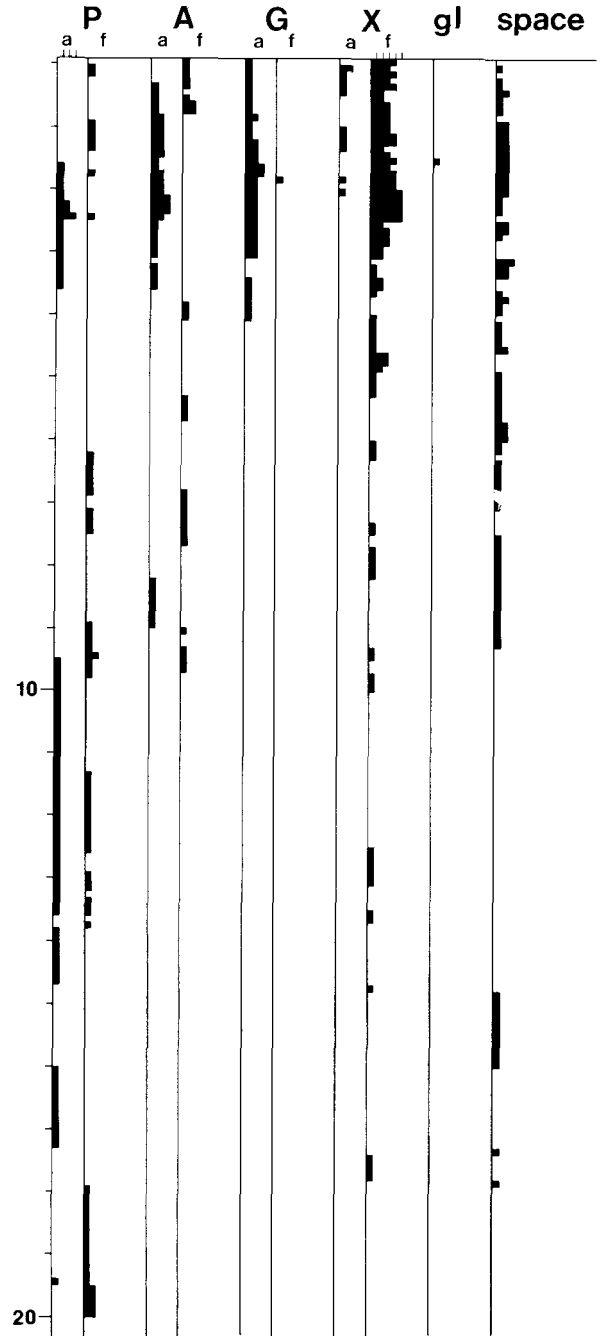

Figure 14. Contacts by the C filopodia. This is a schematic reconstruction of the same cells as in the previous two figures, but showing the relationship between the filopodia extending from the anterior tip of the C cell growth cone and the P cells (*Pa,f*), A cells (*Aa,f*), G cell (*Ga,f*), unnamed cells (*Xa,f*), glial cells (*gl*), and *space*. The C filopodia do not show any significant preferences for contact with the P versus the A cells (see the text for further discussion). *Vertical* and *horizontal scales* are as in Figure 12.

ences between the serial and semiserial reconstructions. First, both of the semiserial reconstructions in which only G was filled with HRP are at an older age where the G growth cone has already begun to extend anteriorly upon the A/P fascicle. The serial section reconstruction is at an age before the G growth cone has directly contacted the A/P fascicle. Second, the contacts of all HRP-filled filopodia with the A/P fascicle were tabulated in the semiserial reconstructions, regardless of where the filopodia originated on the G cell, while in the serial reconstruction only the contacts made by filopodia originating

from the distal 5 μm of the G growth cone were examined. Third, the fixation and preservation of filopodia were superior in the serial reconstruction.

Filopodial contact with unidentified axons (Xa) and glial cells (gl) is minimal and probably unimportant for the guidance of the G cell. The contacts with unidentified filopodia (Xf) are considerable, and we cannot rule out the possible importance of these or a subset of these contacts for the guidance of the G cell. However, these unidentified filopodia come from about 100 different growth cones and axons in the region (within 30 μm of the G growth cone) and do not appear to represent a single source or small number of sources that make a significant contribution to the guidance of the G cell to the A/P fascicle.

The proportion of their length that G's filopodia are not in contact with any substrate is shown in the *space* category. G's filopodia do not contact any substrate for about one-third of their length. This is clearly a composite for all of the filopodia; there are examples at both extremes where filopodia are in continuous contact with a substrate, and alternatively where they seem suspended in space along their entire length.

The last schematic of the serial section reconstruction (Fig. 14) shows the contacts of C's filopodia with the same categories as in Figures 12 and 13. The C growth cone extends across the posterior commissure on the G cell axon and growth cone. Only after the C cell reaches the A/P fascicle does its growth cone change substrates. In this reconstruction the C cell growth cone has not yet left the G cell. The C growth cone and filopodia that were within the region of the reconstruction were identified. Since the C growth cone was in continuous contact with G, only the reconstruction of filopodial contacts is shown (Fig. 14). Comparison between the Ps (Pa,f) and the As (Aa,f) shows no differences for up to 10 μm, but from this point on C's filopodia seem to prefer the Ps (Fig. 14). This is difficult to interpret for two reasons. First, the number of filopodia beyond 10 μm is very small—only two. Second, since C has clearly followed G across the posterior commissure to the A/P fascicle, the initial contacts of the C filopodia may reflect their preference for the G cell. That is, C may be contacting the As and the Ps fortuitously because C is still following G, which is now contacting the A/P fascicle and showing a striking preference for the Ps.

The main results of the serial section reconstruction can be summarized as follows. (*i*) Only P filopodia (two) insert into the G growth cone and induce coated vesicles (Fig. 11). (*ii*) The G growth cone is contacted selectively by P filopodia as compared to A filopodia (Fig. 12). (*iii*) G's filopodia show a significant preference for contact with the A/P fascicle and within the fascicle for the P axons and not the A axons (Fig. 13). (*iv*) C's filopodia do not show this preference for contact with the P axons.

Discussion

In our previous work we described in detail the divergent choices made by the growth cones of the first six progeny of NB 7-4 (Raper et al., 1983a). In particular, we focused our attention on the pathway choices made by the growth cones of the sibling G and C cells. These two cells extend across the posterior commissure on the Q1 fascicle to a position in the contralateral neuropil, that we have called their "choice point," where they grow in opposite directions, G extending anteriorly and C extending posteriorly. The most obvious feature of the choice point at the time that G grows anterior was found to be the A/P fascicle containing four axons: the A1, A2, P1, and P2 cells (Raper et al., 1983b).

The G growth cone extends anteriorly upon the A/P fascicle, whereas the growth cones of its siblings (Q1, Q2, C, Q5, and Q6) make other cell-specific pathway choices. These observations on selective fasciculation during neuronal development

led us to propose the "labeled pathways" hypothesis (Goodman et al., 1982; Raper et al., 1983b). 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 extends upon ideas that have been developing since Cajal's day, as discussed previously (e.g., Raper et al., 1983a, b).

The ultrastructural analysis demonstrates that the G growth cone shows a significant preference for contact with the A/P fascicle as compared to the other longitudinal axon fascicles. Within the A/P fascicle, the tip of G's growth cone prefers to contact the P axons as compared to the A axons. The ultrastructural analysis of the filopodial contacts before the G growth cone reaches the A/P fascicle shows that (*i*) selective contact between the G filopodia and the P axons precedes the choice made by the G growth cone to extend selectively along the P axons within the A/P fascicle; and (*ii*) selective contact between the P filopodia and the G growth cone also predicts the choice made by the G growth cone to extend along the P axons and not the A axons. These results support the labeled pathways hypothesis.

An intriguing observation concerns the two P filopodia that were found to insert into the G growth cone and induce coated pits and vesicles (selective filopidial insertion is discussed below). It is one of these two P filopodia that the tip of the G growth cone is in constant contact with as it reaches the P axons in the A/P fascicle. It seems likely that the tip of the G growth cone is extending along this P filopodium toward the P axons. Thus, just as the filopodia from a growth cone may direct the growth cone by their adhesive interactions with other cells, so the filopodia from other cells may be an important substrate for the adhesion and extension of the growth cone toward those cells.

We previously reported on the discovery of a specific interaction between some of the earliest developing growth cones in the grasshopper embryo as revealed by TEM serial section reconstructions (Bastiani and Goodman, 1984a). Numerous filopodia from an identified growth cone (MP1) insert deep within another identified growth cone (pCC), inducing the formation of coated pits and vesicles. This interaction is highly specific, since filopodia from other nearby growth cones which contact the surface of the two interacting neurons neither penetrate them nor induce coated vesicles.

In the present study, we have observed selective filopodial insertion and the induction of coated pits and vesicles from P filopodia into the G growth cone. Here, too, the interaction is highly specific, since filopodia from many other nearby growth cones and axons which contact the surface of the G growth cone neither penetrate it nor induce coated vesicles. Just as the data on selective filopodial adhesion suggest that axons in different bundles have different cell surfaces that filopodia can distinguish among, so the data on selective filopodial insertion suggest that the filopodia from different neurons have different cell surfaces that growth cones can distinguish among.

The observation of filopodial insertions is not limited to a few early growth cones (MP1 and pCC; Bastiani and Goodman, 1984b) and a few later growth cones (P and G) in the grasshopper CNS. Rather, we have observed this phenomenon between many different identified growth cones at different stages of development in the grasshopper. The presence of filopodial insertions is also not limited to grasshopper growth cones. Vaughn and Sims (1978) describe filopodia-like processes from developing axonal collaterals in mouse spinal cord that are associated with coated pits in postsynaptic cells. They also suggest that "molecular information" may be passed between cells via these interactions and in their case induce changes in cell surface membranes appropriate for specific synaptogenesis.

Nordlander and Singer (1982) have seen profiles in the growth cones of amphibian embryos which are similar to those seen in Figure 11C, but they did not identify the source of these structures or observe whether coated pit formation occurred at the tips. Slavkin and Bringas (1976) describe long filopodia-like processes from mesenchymal cells protruding into epithelial cells of the developing tooth germ during the suggested inductive interaction between these two tissues. Thus, the specific interaction of filopodial insertion and induction of coated vesicles may be a general mechanism underlying many inductive events during development.

What might be the function of these selective filopodial insertions? We have already suggested that selective filopodial adhesion is the mechanism of cell recognition that guides a growth cone onto a particular axon fascicle. However, growth cones typically make a series of such selective fasciculation choices on the way to their appropriate target, sequentially getting onto and then off of particular axon bundles. One attractive hypothesis is that the selective adhesion of filopodia is a dynamic process such that the adhesive properties of a growth cone change during the course of their navigations along the scaffold of axon fascicles. We speculate that interactions involved in navigating through one choice point might induce a cell to change its expression of cell surface molecules involved in (*i*) the filopodial adhesion at that choice point or (*ii*) the filopodial adhesion at a subsequent choice point. How, though, does the biochemical machinery in the cell know where the growth cone is and what it is doing? One possibility is that the specific events described here might mediate such inductive changes by signaling to the cell's machinery via receptor-mediated endocytosis. We propose that when the G growth cone encounters the P axons, the selective adhesion of the G filopodia onto the P axons guides the G growth cone onto the A/P fascicle. The selective insertions of the P filopodia into the G growth cone induces changes in the G growth cone that either enhance this adhesive interaction with the P axons or prepare it for subsequent encounters.

Several questions concerning our interpretation of the results should be addressed here. First, the small sample size of five semiserial and one serial reconstruction, each at a slightly different age, requires that any conclusions be cautiously presented. However, the consistency of these results with our previous light level results (Raper et al., 1983a, b) and with the experimental results presented in the next paper (Raper et al., 1984) further supports our conclusions. Second, we must ask whether the observation of selective filopodial and growth cone contacts can be explained by some other mechanism rather than evoking selective filopodial adhesion to the differentially labeled P axons and A axons. We have considered a variety of mechanisms based solely on temporal and/or spatial relations, or on global chemotactic gradients, and have been unable to come up with an alternative hypothesis that satisfactorily explains our results without evoking much more complex phenomena. For example, the preference of G's growth cone and filopodia for the P cells versus the A cells cannot simply be explained by assuming a preference for growth cone membrane versus axonal membrane. As shown in Figures 7 and 8 and elsewhere, G's growth cone and filopodia selectively contact the P cells regardless of the proximity and location of the A growth cones. Moreover, our model based on differential filopodial adhesion is consistent with extensive studies *in vitro* (e.g., Bray, 1982; Letourneau, 1982). Third, we are trying to interpret a static ultrastructural picture to explain the dynamic processes of filopodial interactions and selective fasciculation by growth cones. However, we have intentionally chosen to examine the ultrastructure at the time when we feel that the most interesting interactions and choices are taking place, but out of necessity this initial bias has biased and restricted our results.

The results of the ultrastructural analysis presented here support the "labeled pathways" hypothesis and emphasize the role of selective filopodial contact in the guidance of growth cones. In addition, they have suggested two different forms of selective filopodial contacts: (*i*) adhesion to the cell surface and (*ii*) insertion into the cell and induction of coated vesicles. It appears that G's filopodia can distinguish the A/P fascicle from other longitudinal axon bundles and, moreover, within this fascicle 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. G's filopodia appear to direct the G growth cone off of the Q1 fascicle and onto the A/P fascicle even in the absence of any other substrate between the two fascicles.

The specificity of these interactions implies the presence of many different molecular labels on growth cones and axons in the developing neuropil. At this stage of development, G's growth cone is within 30 μm, and, thus, within filopodial grasp, of nearly the entirety of the contralateral neuropil consisting of about 100 different growth cones and axons in about 25 different axon fascicles (e.g., Fig. 1). Yet G shows a preference for contact with the A/P fascicle. The unexpected specificity shown by G's filopodia and growth cone for the P axons versus the A axons implies that there is not a single label on the A/P fascicle, but rather that the Ps are labeled differently than the As. The same conclusion is supported by the highly selective filopodial contacts between P (versus A) filopodia and the G growth cone, not the least of which is the filopodial insertion and induction of coated vesicles by P filopodia.

In the companion paper (Raper et al., 1984), the labeled pathways hypothesis is tested experimentally by specific axon ablations. The results further support the hypothesis.

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