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PATHFINDING BY NEURONAL GROWTH CONES IN GRASSHOPPER EMBRYOS

II. Selective Fasciculation onto Specific Axonal Pathways[1]

JONATHAN A. RAPER,[2] MICHAEL BASTIANI, AND COREY S. GOODMAN

Department of Biological Sciences, Stanford University, Stanford, California 94305

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Abstract

In the previous paper (Raper, J. A., M. Bastiani, and C. S. Goodman (1983) J. Neurosci. *3:* 20–30) we showed that the growth cones of two sibling neurons, the G and C cells, follow the same route in the developing grasshopper neuropil until they reach a stereotypic choice point. Here their growth cones diverge from each other as G turns and extends anteriorly and C turns and extends posteriorly. In this paper we show that the G and C growth cones fasciculate and extend in opposite directions upon a specific bundle of four axons. This occurs even though many other axons are within filopodial reach of the G and C growth cones. We identified the four neurons (the A1, A2, P1, and P2 cells) whose axons form the bundle that G and C extend upon by filling individual axons with Lucifer Yellow and viewing the filled cells in living embryos and by filling individual neurons with HRP and reconstructing the axon bundle from electron micrographs. The G neuron extends anteriorly in the bundle containing these four axons; the C neuron extends posteriorly in the same bundle only after several other axons have joined in. These results suggest that the growth cones of the G and C neurons are determined to recognize and extend upon labeled axons, leading us to propose the "labeled pathways" hypothesis.

In the previous paper (Raper et al., 1983) we described the divergent choices made by the growth cones of two sibling neurons, the G and C cells, in the grasshopper embryo. The G and C growth cones follow the same route across the posterior commissure of the second thoracic ganglion until they reach a specific "choice point" on the contralateral side of the developing neuropil. At this choice point they diverge; G's growth cone extends anteriorly, while C's growth cone later extends posteriorly.

In the present study our aim has been to identify the environmental cues which guide the G and C growth cones to this choice point and then determine the routes they will take as they leave it. In principle, the relevant information could reside in the neuropil in the form of global gradients (e.g., Sperry, 1963; Trisler et al., 1981) or as localized biochemical and/or structural heterogeneities (e.g., Silver and Robb, 1979; Singer et al., 1979; Lance-Jones and Landmesser, 1981a, b; Bate, 1976b; Ho and Goodman, 1982). In this paper we show that the G and C growth cones diverge upon a discrete bundle of axons initially formed by the processes of four identified neurons. The G growth cone extends anteriorly upon these four axons; the C growth cone extends posteriorly only after several other axons have joined the bundle. Our finding suggests the "labeled pathways" hypothesis, a notion similar to that discussed by Ghysen and Janson (1980), whereby the growth cones of later differentiating neurons are programmed to choose between and elongate upon the axons of specifically labeled, earlier differentiating neurons.

Materials and Methods

We can view the cell bodies and, in some cases, the axons and growth cones of identified neurons in the grasshopper embryo using a Zeiss compound microscope with Nomarski interference contrast optics and a Leitz × 50 water immersion lens. The cell bodies and processes can be injected with the fluorescent dye Lucifer Yellow, and their complete cellular morphology then can be

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[2] To whom correspondence should be addressed.

viewed in the living embryo with blue light (for details see Raper et al., 1983). As many as five neurons can be alternately filled and viewed in the same living preparation, an absolute requirement for the observations we report here.

Some neurons were injected with horseradish peroxidase (HRP; Boehringer Mannheim Grade I, lyophilized) so that the positions of their growth cones could be studied by transmission electron microscopy. HRP was iontophoresed into cell bodies by 1.5- to 2.0-nA positive current pulses (Gilbert and Wiesel, 1979). Sometimes the HRP was dissolved in a mixture of 0.15 M sodium acetate and 1% Lucifer Yellow. By filling impaled neurons first with HRP, then with Lucifer Yellow, and visualizing the Lucifer Yellow in blue light, we could verify that we had filled the correct neuron. Injected neurons sat in (hypotonic) physiological saline (described in Raper et al., 1983) between 5 and 20 min before fixation in 2% glutaraldehyde and 2% paraformaldehyde dissolved in Millonig's buffer (Bate, 1976a). The HRP was developed using the 3,3-diaminobenzidine and glucose oxidase reaction mixture of Watson and Burrows (1981). The embryos were osmicated and prepared for electron microscopy according to the method of Bate (1976a).

The axons of the large subset of neurons shown in Figure 3 were stained in whole mount embryos using the I-5 monoclonal antibody and an HRP-conjugated second antibody as developed and described by S. Chang, R. K. Ho, and C. S. Goodman (manuscript in review).

The growth cone shown in Figure 4 was filled with Lucifer Yellow and stained using an antibody to Lucifer Yellow and an HRP-conjugated second antibody as developed and described by Taghert et al. (1982).

The scanning electron micrograph in Figure 2 was made by first embedding the embryo in plastic (Bate, 1976a), sectioning the block until the desired area was reached, putting the block in a solution of 0.5% sodium methylate in 100% methanol for 2 days to etch away the plastic, washing the block in 100% methanol for 1 day, and then critical point-drying and gold-coating the block as in normal scanning electron microscopy (SEM) (John Mais, personal communication).

Results

The first six progeny of the identified neuronal precursor cell, neuroblast (NB) 7-4, are the Q1, Q2, G, C, Q5, and Q6 neurons (Raper et al., 1983). Q1's growth cone pioneers one of the first axonal pathways across the T2 ganglion in the posterior commissure; its morphology at this early time is shown in Figure 1*A*. The Q1 growth cone, when viewed with Nomarski optics in white light, appears to cling to the thin basement membrane (Bate and Grunewald, 1981) which covers the dorsal surface of the ganglion. It falls off the membrane after reaching the axon of its contralateral homologue at the ganglionic midline and continues growing upon its homologue's axon.

The Q2, G, C, Q5, and Q6 growth cones all proceed in sequence across the posterior commissure within the axon bundle established by Q1, even though several other bundles have been established by this developmental time (Fig. 2). When any subset of the Q1 through Q6 cells are filled simultaneously with Lucifer Yellow, their axons are so close to each other that they are indistinguishable in the fluorescence microscope. Since we have found that the filopodia of growth cones (diameter ~0.15 μm) can be resolved under these conditions, our findings provide strong evidence that the growth cones and axons of the sibling Q1, Q2, G, C, Q5, and Q6 neurons are in very close apposition within a single bundle of the posterior com-

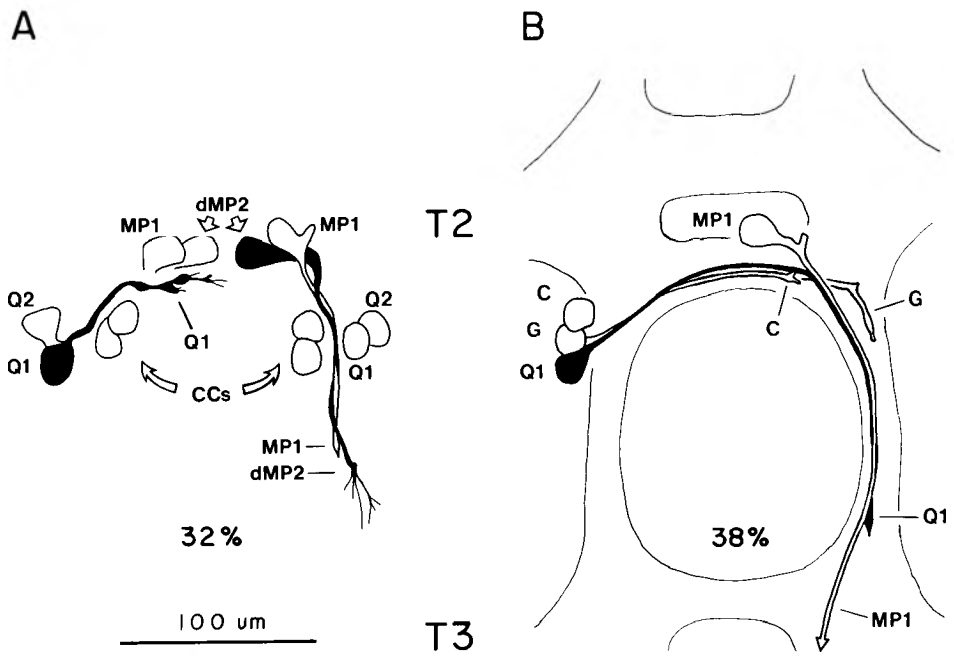


Figure 1. Q1 follows an identified axon posteriorly. *A*, A camera lucida tracing of a Q1 cell and the contralateral dorsal MP2 (dMP2) and MP1 cells filled with Lucifer Yellow. At 32%, Q1's axon is just reaching the ganglionic midline, while the dMP2 and MP1 axons have already pioneered an axon bundle in the ganglionic connectives. Another bilateral pair of neurons, the CC cells, are also shown. *B*, By 38%, Q1 has crossed the ganglionic midline and turned posteriorly upon the MP1 and dMP2 axons. G's growth cone does not turn posteriorly upon the dMP2 but proceeds to a more lateral position in the neuropil.

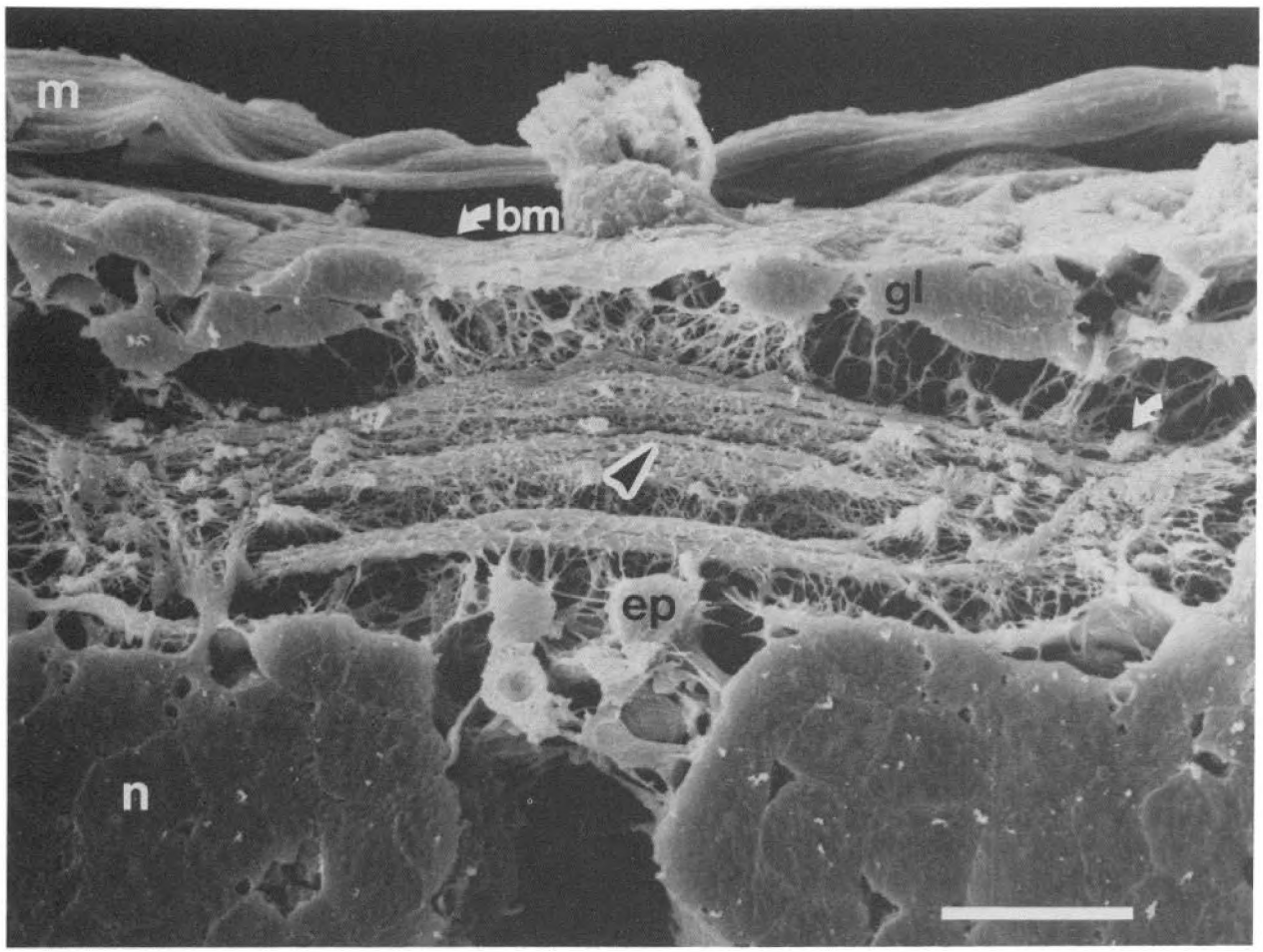


Figure 2. Several discrete axon bundles cross in the posterior commissure. Scanning electron micrograph of a cross-section of the posterior commissure in a 41% embryo. A *black arrowhead* indicates the bundle in which the axons of the early progeny of NB 7-4 travel. A *white arrow* indicates the axon bundle in the lateral neuropil upon which the G and C growth cones diverge and extend in the ganglionic connectives. *m*, muscle sheet over the dorsal surface of the ganglion; *bm*, basement membrane covering the dorsal surface of the neuroepithelium; *gl*, glial cells; *ep*, epidermal cells; *n*, neurons. *Scale bar*: 20 μm.

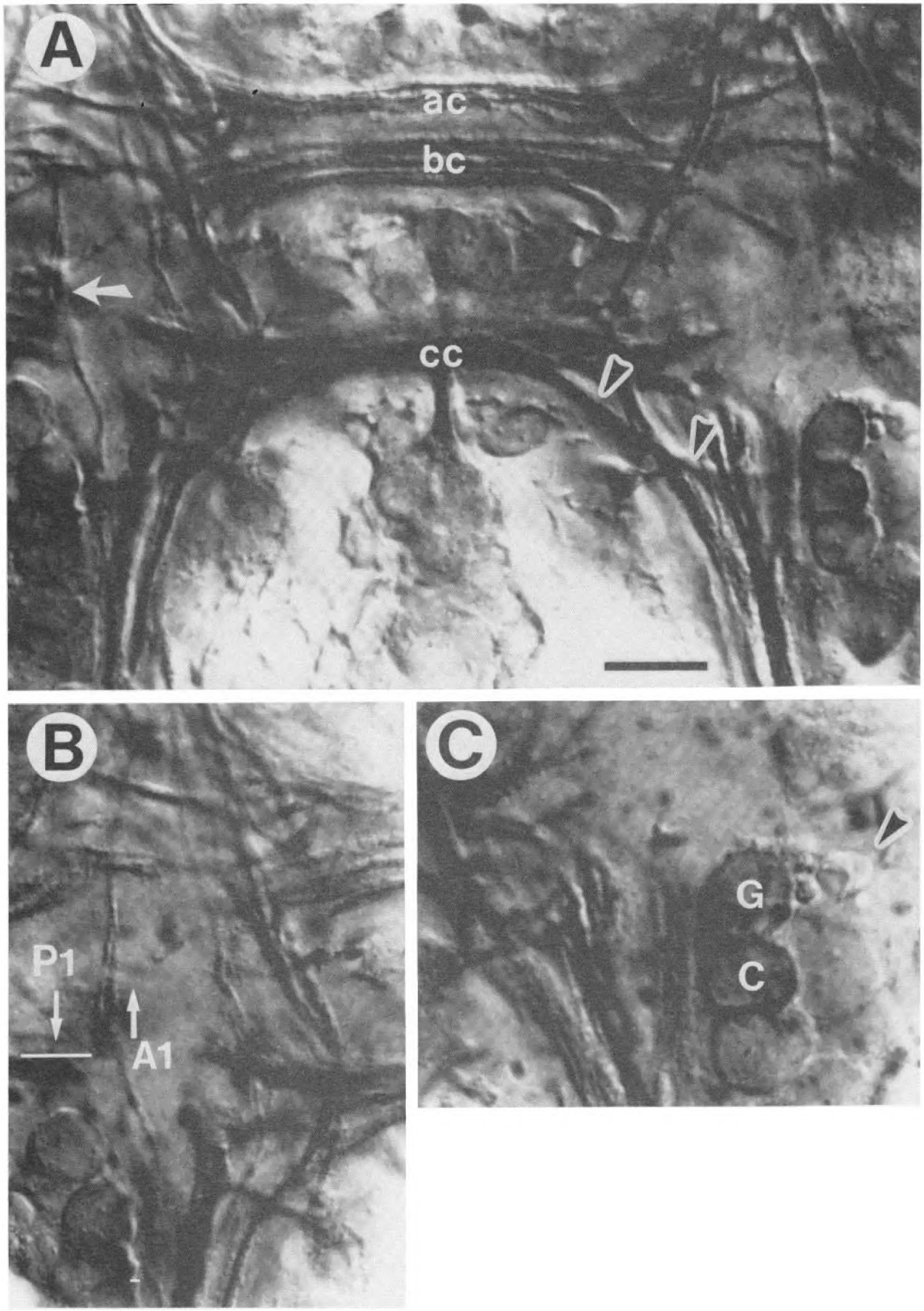
missure. Several of the axon bundles in the posterior commissure are shown in the scanning electron micrograph of Figure 2, including the bundle containing the axons of the NB 7-4 progeny.

As the Q1 and Q2 cells turn posteriorly in the contralateral neuropil, their growth cones can be seen with simultaneous Lucifer Yellow fills to cling to the axons of another pair of neurons, the MP1 and dorsal MP2 cells (Fig. 1*B*) (Bate and Grunewald, 1981; Goodman et al., 1982; Taghert et al., 1982). This is not true, however, for the G and C growth cones. They grow past the location where Q1 and Q2 turn posteriorly and continue extending to a more distal location in the neuropil. It is here that they pause before G's growth cone turns and elongates anteriorly.

By filling the G neuron with Lucifer Yellow just after its growth cone has turned anteriorly, visualizing its growth cone with fluorescence, and then examining the same location in white light with Nomarski optics, we find that G's growth cone elongates in apposition to a discrete axon bundle in the neuropil. This bundle (Fig. 2, *white arrow*) and many others are stained in a whole mount preparation (Fig. 3) treated with the I-5 monoclonal antibody developed by S. Chang, R. K. Ho, and C. S. Goodman (manuscript in review). The bundle is in the dorsal, lateral margin of the neuropil and extends both anteriorly and posteriorly into the lateral margin of the ganglionic connective.

The morphology of G's growth cone just before the bundle has formed is shown in Figure 4. The growth cone of one of the neurons (A1, see description below) which pioneers the bundle has just grown anteriorly past G. The G growth cone has discrete active sites (defined and described in Raper et al., 1983) which are aligned with axons or bundles of axons passing at right angles near the G growth cone. Active sites whose filopodia reach near the axon pioneering the lateral bundle are indicated with *arrows*. One of these sites of profuse filopodial extension is directed posteriorly along the bundle pioneer. Another has many filopodia directed anteriorly near the same axon. The next most medial active site has at least one filopodial process extending to the axon pioneering the lateral bundle.

We have identified the neurons whose axons form the lateral bundle upon which the growth cones of G and C diverge. We penetrated individual axons with microelec-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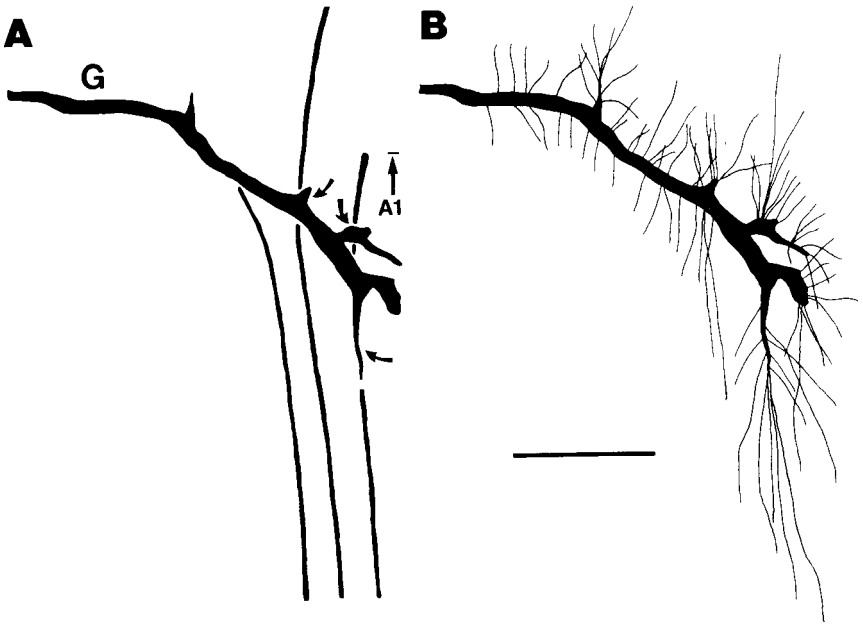

Figure 4. Morphology of the G growth cone before it turns anteriorly. Camera lucida tracings of a G cell filled with Lucifer Yellow and visualized with an anti-Lucifer Yellow antibody reacted with an HRP-conjugated second antibody. *A*, The G growth cone's profile is shown without its filopodia drawn in. Active sites (see the test) are aligned with axons passing near the growth cone. The growth cone of the A1 cell (see the test and Fig. 5) has just grown anteriorly through the point at which G will turn anteriorly. The direction in which it is extending is indicated by an *arrow*. *B*, The same growth cone with the filopodia added. Filopodia from three active sites (*curved arrows* in *A*) reach the vicinity of the A1 axon.

trodes (the axons are 1 to 2 μm in diameter), filled them with Lucifer Yellow, and viewed the filled neurons in living whole mount embryos.

The axon bundle upon which G elongates is formed initially by two pairs of axons (Fig. 5). One pair originates from the A1 and A2 cells which are located in the next posterior segment from that containing the G and C neurons. The A1 and A2 cell bodies are on the opposite side from the axon bundle they help pioneer. The growth cones of these two anteriorly growing axons meet (Fig. 2*B*) and grow upon a pair of posteriorly extending axons which originate from the P1 and P2 cells. The P1 and P2 cell bodies are located in the same segment as G and C and are on the same side as the axon bundle they help pioneer. Judging by their very different positions in the ganglion, it is likely that the A and P cell pairs are not born from the same or homologous neuroblast and are therefore unrelated. The A1 and A2 cells, as well as the P1 and P2 cells, are likely to be sibling pairs since their cell bodies cluster together, their axons follow one another, and they are dye coupled to each other. In both cases, the growth cone of one cell (designated A1 or P1) leads the other (A2 or P2) by about 50 μm. The A1 and P1 growth cones grow along the dorsal basement membrane in opposite directions. They meet at about the center of the second thoracic segment and fasciculate upon one another. Their axon bundle then falls off of the basement membrane.

We have correlated the behavior of the G and C growth cones with the arrival and departure times of the A1, A2, P1, and P2 axons at the choice point where G and C diverge. In more than 20 preparations G was never observed to turn anteriorly before A1's growth cone passed through the choice point. Figure 6*A* shows the morphology of the G neuron just after A1 has passed through the choice point, but before A2 has reached it. Less than 10 hr later, the G growth cone has begun to elongate anteriorly behind the A1 and A2 growth cones (Fig. 6*B*). G's growth cone and axon appear to be in direct contact to A1 and A2 when these cells are filled with Lucifer Yellow and viewed by fluorescence.

We have filled the G neuron with HRP at comparable developmental times (Fig. 7*A*) and examined the relative positions of the G growth cone to the individual fibers within the bundle in five preparations. At this time the G growth cone is in an axon bundle containing four other

Figure 3. Some of the axonal pathways present in the developing neuropil just before the G and C growth cones diverge. A whole mount preparation of a 40% embryo stained by the monoclonal antibody I-5. The neuropil of a ganglionic segment is viewed from the dorsal surface. *A*, One posterior (*cc*) and two anterior (*ac*, *bc*) commissures join the two sides of the ganglion. A parallel pair of anterior-posterior 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 the text and Fig. 5) meeting to form the lateral axon bundle upon which the G and C growth cones will 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*. *Scale bar*: *A*, 20 μm; *B*, 25 μm; *C*, 30 μm.

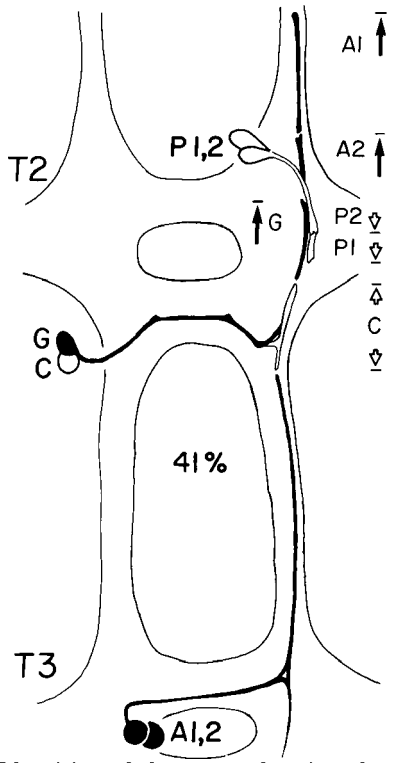

Figure 5. Identities of the axons forming the bundle upon which G and C diverge. A single living preparation in which the neurons displayed were filled with Lucifer Yellow and then traced with the aid of a camera lucida. Anteriorly extending axons are the *profiles*. The A1 and A2 axons ascend from the next posterior (T3) ganglion through the T2 neuropil contralateral to the G and C cell bodies. The directions in which the growth cones extend are indicated by *solid arrows*; *horizontal lines* mark their distalmost tips. Posteriorly extending axons are the *open profiles*. The P1 and P2 axons descend from the anterior portion of the T2 segment through the neuropil contralateral to the G and C cell bodies. The positions of these growth cones are marked with *open arrows* to the side.

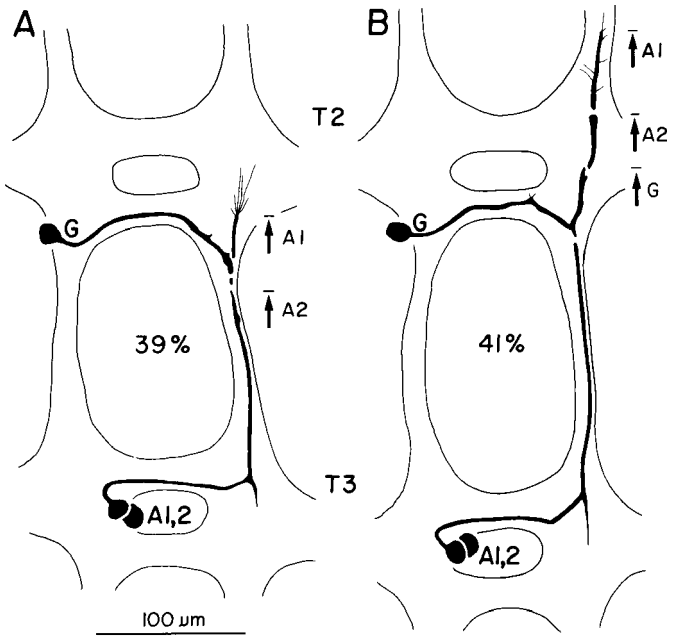

Figure 6. The relative timing of G's anterior extension relative to that of A1 and A2. *A*, Camera lucida tracings showing the relative positions of the G, A1, and A2 growth cones in a 39% embryo. A1 has grown anteriorly past G's growth cone. *B*, G extends anteriorly upon the axons and behind the growth cones of the A1 and A2 neurons. The directions in which the growth cones extend are indicated by *arrows*; *horizontal lines* mark the distalmost tips.

axons (Fig. 8, *A* and *B*). By tracing these four other axons anteriorly and posteriorly from electron micrographs at 1-µm intervals, we have verified that two of the axons belong to A1 and A2 and the other two axons belong to P1 and P2.

C's growth cone begins its posterior extension at about the time that the P1 and P2 axons pass through the choice point. Although the P growth cones are very close to the choice point even as G turns anteriorly, their posterior extension is rather slow, particularly when compared to the more rapid anterior growth of A1 and A2. It can therefore be as long as 10 hr after the A axons pass through the choice point that the P axons reach it. The relative positions of the C, P1, and P2 growth cones are shown before (Fig. 9*A*) and after (Fig. 9*B*) C has made a clear posteriorly directed choice.

Electron micrographs of HRP-filled neurons confirm our observation from Lucifer Yellow fills that 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, and P2 axons (Fig. 8, *C* and *D*). We are not yet certain that we can identify all of the additional profiles in the bundle at this stage. For example, the A1 and A2 axons from two ganglia posterior to the G and C neurons arrive at about the time that C begins extending posteriorly.

There is another potentially important pair of identified neurons which contribute axons to this bundle. The cell bodies of the X1 and X2 neurons lie just lateral to the G and C cell bodies. We do not know yet from which neuroblast they are born, but they do not arise from NB 7-4. The axons from X1 and X2 cross in the posterior commissure and in the contralateral neuropil they join the axon bundle in which G and C are found. The X1 and X2 axons extend posteriorly at a fast rate in advance of C's growth cone (Fig. 10). Thus, in addition to the P cells, the X cells provide axons which C could follow posteriorly.

One final point about the G and C neurons concerns their dye coupling to other cells. By dye coupling, we mean the spread of Lucifer Yellow (450 M_r) from the interior of one cell to another. We have noticed distinctive patterns of dye coupling between the interiors of individually identified neurons at early stages of their differentiation (Goodman and Spitzer, 1979; Goodman et al., 1982; Bate and Grunewald, 1981; Raper and Goodman, 1982). In the grasshopper embryo it is common for related neurons, those born from the same neuroblast, to be dye coupled at early stages. Dye coupling to unrelated neurons is more commonly observed between early differentiating neurons whose growth cones pioneer axonal

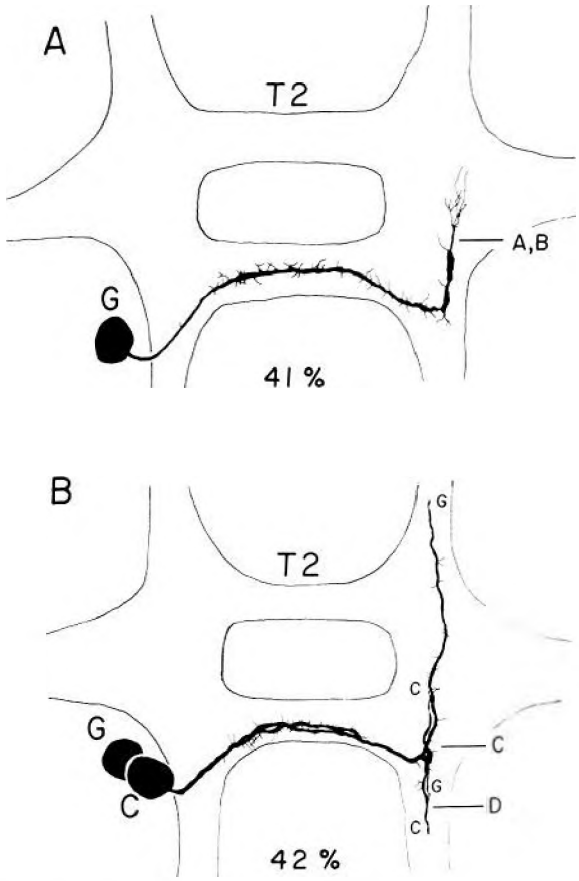

Figure 7. G and C neurons examined by transmission electron microscopy. Camera lucida tracings of HRP-filled neurons in whole mounts before they were thin sectioned. *A*, A G neuron which has just begun to extend anteriorly; *B*, G and C neurons slightly later in development when G's growth cone is leaving the T2 neuropil and the C cell extends symmetrically in both the anterior and posterior directions. *Horizontal lines* mark the levels of sections in the four panels (*A* to *D*) of Figure 8.

pathways and other neurons in their environment. Before G and C reach their choice point, they are strongly dye coupled to the Q1 and Q2 neurons. Shortly thereafter, Q1 and Q2 die, leaving G and C strongly coupled to Q5 and Q6; these cells are strongly dye coupled to their contralateral homologues. In the hundreds of embryos in which we have filled either the G or C cell bodies with Lucifer Yellow at about the time their growth cones diverge, we have only occasionally observed them to be dye coupled to unrelated neurons. However, these few cases are particularly interesting because they involve the axons we have described in the lateral bundle. In 2 embryos, the G cell was dye coupled to the A cells; in 4 embryos, the C cell was dye coupled to the P cells; and in 3 embryos, the C cell was dye coupled to the X cells. The specific dye coupling we occasionally detect between these axons probably reflects their intimacy in the neuropil and indicates that their cell interiors are at least sometimes in communication with each other. Furthermore, this supports the notion that the C growth cone is following either the P axons, the X axons, or possibly both sets of axons.

Discussion

Our goal was to identify the environmental cues which guide the G and C growth cones along their appropriate routes and through their stereotypic choice points in the developing neuropil. In a reliable sequence, the G and C growth cones follow the axons of specific earlier differentiating neurons. Our findings are summarized in Figure 11. The first two progeny of NB 7-4, Q1 and Q2, extend to the ganglionic midline in an axonless environment; Q1 is the first growth cone to pioneer the pathway followed by its later siblings. The Q1 growth cone meets the growth cone of its contralateral homologue Q1 at the midline and continues growing along its homologue until it reaches the posteriorly directed axons of the MP1 and dorsal MP2 (dMP2). Here Q1 and, shortly thereafter, Q2 turn posteriorly, their growth cones clinging to the MP1 and dMP2 axons. The growth cones of G and C extend across the midline upon the axons of the Q1 and Q2 neurons until G and C reach a distal position in the contralateral neuropil. Here G extends anteriorly following the axons of two identified neurons, the A1 and A2 cells. G continues following A1 and A2 anteriorly past the point at which the P1 and P2 axons join the bundle. Later, C extends posteriorly behind the P1, P2, X1, and X2 growth cones in the same discrete bundle of axons already joined by G. The next two progeny of NB 7-4, Q5 and Q6, follow the axons of G and C across the ganglionic midline. They turn anteriorly in a more medial portion of the neuropil. These results suggest that specific axons or bundles of axons serve as the environmental cues which guide the growth cones of Q1, Q2, G, C, Q5, and Q6.

Specific axon tracts have been implicated in the guidance of growth cones by experiments involving surgically and genetically transplanted axons. Studies on both vertebrate and invertebrate neurons demonstrate that axons entering the central nervous system in abnormal locations are able to find and grow in specific axon tracts. Transplanted Mauthner cell axons and optic nerve axons find and grow in separate and specific axon tracts in the frog central nervous system (Constantine-Paton and Capranica, 1975, 1976; Katz and Lasek, 1979, 1981). Similarly, axons from transplanted mechanosensory neurons find and grow in a particular axon tract in the grasshopper central nervous system (Anderson, 1981). A striking example of a similar phenomenon is provided by studies on homeotic mutants of *Drosophila* in which sensory axons that enter the segmental nervous system by a genetically altered route nevertheless extend and grow in their appropriate tracts in the CNS (Ghysen, 1978). These and further results led Ghysen and Janson (1980) to suggest that the growth cones of sensory axons were able to recognize and follow "a specific trail" in the nervous system. They suggest that these trails might be specifically labeled axon pathways established by early differentiating neurons, perhaps analogous to the neurons which pioneer axon pathways in the grasshopper peripheral and central nervous system (Bate, 1976b; Keshishian, 1980; Bate and Grunewald, 1981; Goodman et al., 1982; Ho and Goodman, 1982; Taghert et al., 1982).

Our findings on pathfinding by neuronal growth cones

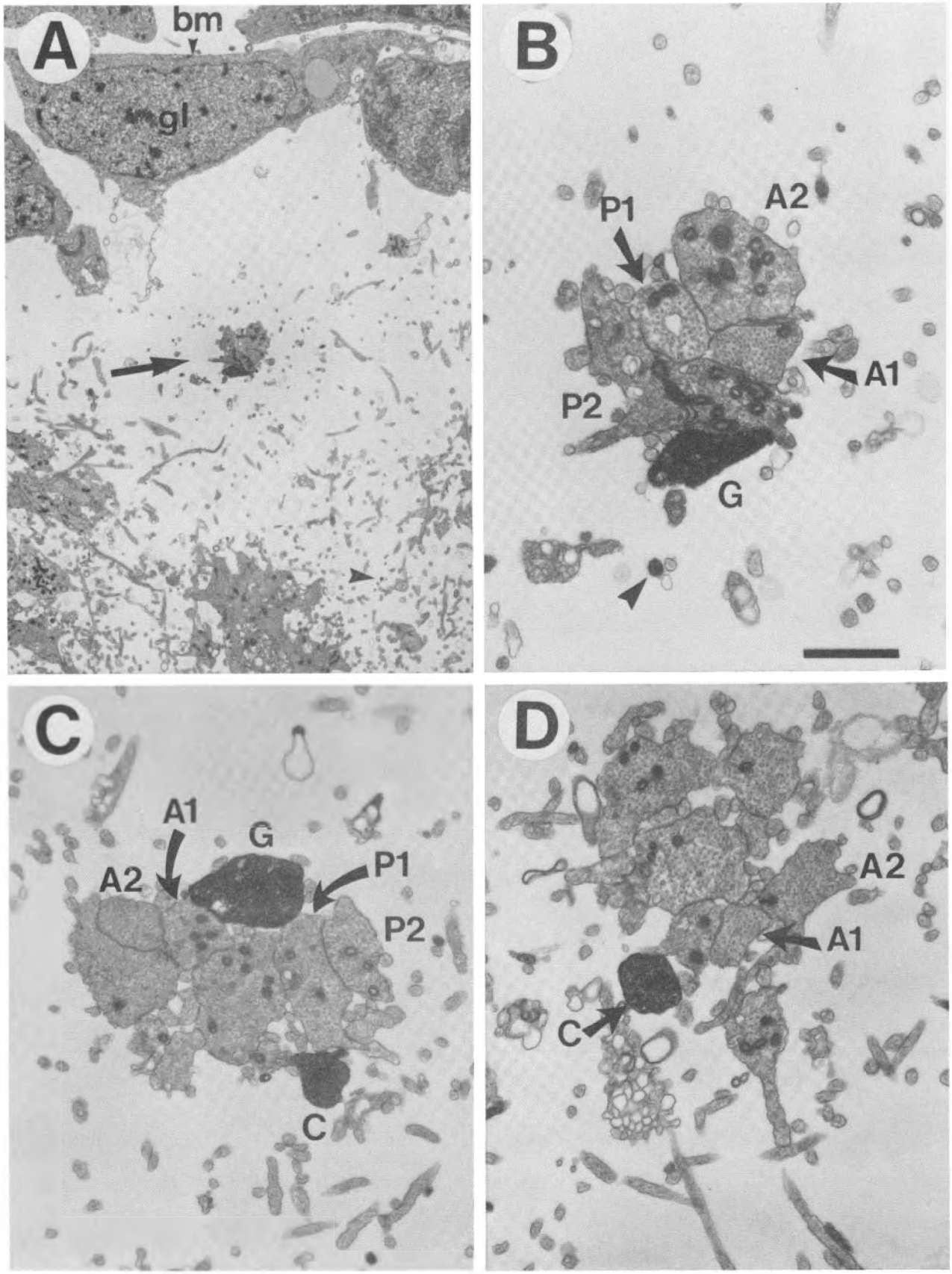

Figure 8. Electron micrographs of the axon bundle in which the G and C growth cones diverge. Cross-sections of embryonic neuropil; dorsal is to the *top* of the figure and the ganglionic midline is to the *left*. *A*, Low magnification view centered upon the axon bundle (*large arrow*) in which G extends anteriorly and C extends posteriorly. G is the ventral axon profile filled with reaction product. The location of one of its many filopodia is marked (*arrowhead*). *bm*, basement membrane; *gl*, glial cell. *B*, Higher magnification view of the axon bundle. The procedure for identifying axons is described in the text. Many filopodia surround the bundle, including one of G's (*arrowhead*). *C*, View of another older preparation in which both the G (dorsal axon) and C (ventral axon) neurons were filled with HRP. There are an additional five axons belonging to unidentified neurons. *D*, The same bundle sectioned further posteriorly. The P1, P2, and G profiles have dropped out. There are five axons belonging to unidentified neurons at this level also, but only some of them correspond to profiles in *C*. *Scale bar*: *A*, 5 μm; *B* to *D*, 1 μm.

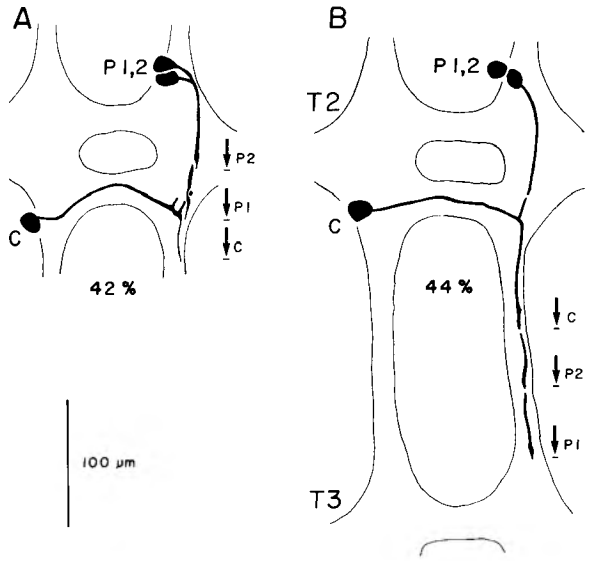

Figure 9. The relative timing of C's posterior extension to that of P1 and P2. *A*, At 42%, C's growth cone has branched and extends for a short distance in both the anterior and posterior directions. The P1 growth cone has just reached the point at which G and C diverge. *B*, A half day later, C's posteriorly directed process extends near or upon the axons and behind the growth cones of the P1 and P2 neurons. In this preparation C was dye coupled to the P1 and P2 cells. The directions in which the growth cones extend are indicated by *arrows*; *horizontal lines* mark their distalmost tips.

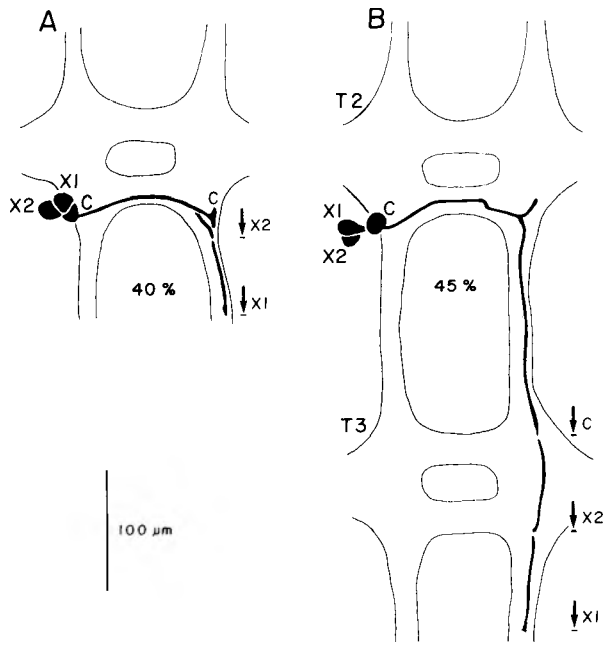

Figure 10. The relative timing of C's posterior extension to that of X1 and X2. *A*, At 42% of development, X1 and X2 have already turned and begun to extend posteriorly, while C extends for only a very short distance in both the anterior and posterior directions. *B*, One day later, C extends posteriorly near or upon the axons and behind the growth cones of the X1 and X2 neurons. The directions in which the growth cones extend are indicated by *arrows*; *horizontal lines* mark their distalmost tips.

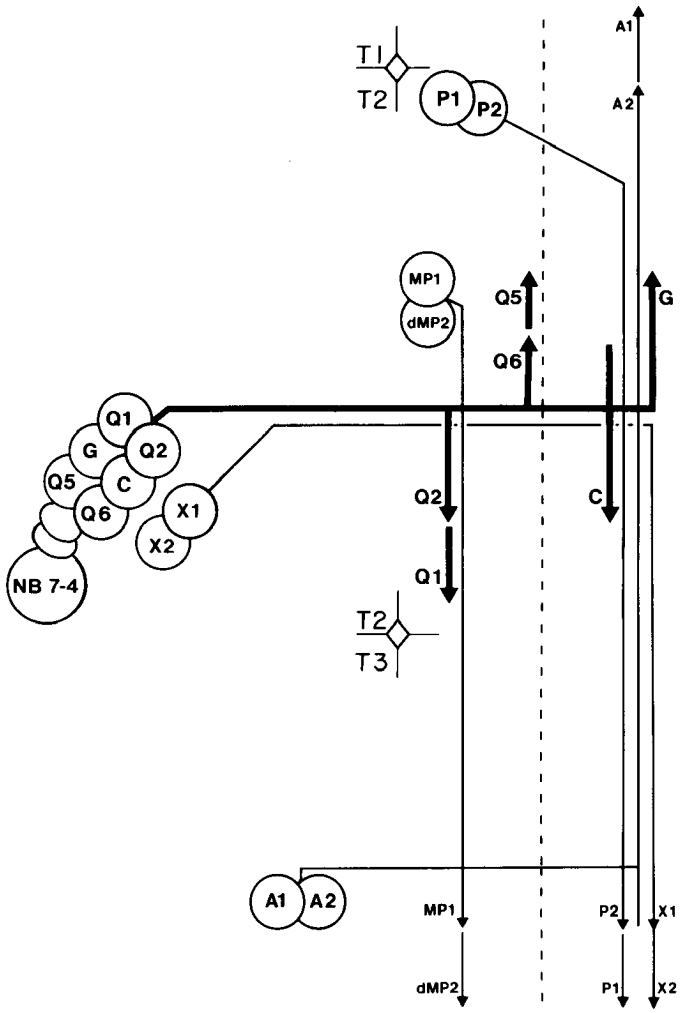

Figure 11. The growth cones of the first six progeny of NB 7-4 extend upon specific axonal pathways in the developing neuropil. After crossing the ganglionic midline, the Q1 and Q2 axons turn posteriorly upon the MP1 and dorsal MP2 (dMP2) axons. The subsequent progeny of NB 7-4 cross the posterior commissure upon the axons of Q1 and Q2. When it reaches the contralateral neuropil, G extends anteriorly upon the A1 and A2 axons. C extends posteriorly near or upon the P1, P2, X1, and X2 axons. Q5 and Q6 extend anteriorly near a different, unidentified axon bundle (*dashed line*).

in grasshopper embryos are consistent with these previous studies on surgically and genetically transplanted axons. For example, the G neuron behaves as if it were programmed to follow the Q1 and Q2 axons across the posterior commissure and then follow the A1 and A2 axons anteriorly in the connective.

We therefore propose the "labeled pathways" hypothesis (Goodman et al., 1982) whereby:

i. a small number of early differentiating neurons pioneer a stereotyped array of axonal pathways;
ii. these axonal pathways are labeled differentially, most likely on their cell surfaces;
iii. the growth cones of later differentiating neurons are programmed to choose between and elongate upon these specifically labeled axonal pathways.

This hypothesis includes the notion that filopodia are

actively involved in sampling the environment (e.g., Bray, 1982) and thus that growth cones can sample several different axonal pathways at their choice points.

The labeled pathways hypothesis implies that a neuron's morphology arises by an ordered sequence of pathway choices made by its growth cone as it is confronted with multiple pathways. As a given neuron's growth cone jumps between the labeled axons it is determined to follow, its morphology could assume a more complex and functionally more meaningful configuration than any of the individual axons it uses for navigation. A neuron's final morphology might then be a collection of joined line segments, with each segment representing a period when the cell's growth cone followed a single pathway and with each joint between segments representing an instance when the growth cone left one pathway to join another.

One refinement of this hypothesis would be to determine if subsets of axons within a bundle (e.g., A1 and A2 vs. P1 and P2) have labels that distinguish them from each other. We would also like to know to what degree different axons have unique labels or, alternatively, use a combinatorial code of only a few molecules in many places and times in differing combinations and strengths. In this way, a small number of molecules could encode a large number of identities.

One alternative to this proposal is that a given pathway is always delineated by markers extrinsic to the axons it contains. Thus, G's growth cone may not recognize and follow Q1 and Q2 and then turn anteriorly on A1 and A2, but rather may be following the same cues used by Q1, Q2, A1, and A2 in the first instance. Arguing against this possibility, however, is the changing location of the pathway. When A1 and P1 are first pioneering the lateral pathway, they are growing on the dorsal basement membrane. Once they reach each other (just as when Q1 meets its contralateral homologue at the midline), they continue growing on each other's axons rather than the basement membrane. Because axons do not stick to the basement membrane (Bate and Grunewald, 1981), the bundle drops off of the membrane. By the time the G growth cone turns anteriorly on the A1 and A2 axons, the bundle is already displaced over 15 μm from the basement membrane, and the membrane itself is covered with glia by this time (M. Bastiani, J. A. Raper, and C. S. Goodman, manuscript in preparation). These observations support the notion that the G growth cone is not following local cues on the basement membrane that previously guided A1, A2, P1, and P2 but rather is following the axon bundle itself. This argument does not exclude G's guidance by more complicated local interactions in three dimensions or by global biochemical gradients. Given the precision with which a complex array of axonal pathways is formed, it seems unlikely that global gradients alone could guide growth cones through their specific choice points.

One final point is the question of polarity. How can the G growth cone distinguish at the choice point between the anterior (correct) and posterior (incorrect) directions in which to follow the A axons? There are four alternatives: (*i*) the A axons could themselves contain polarity information on their surfaces; for example, in the form of "old" vs. "new" membrane recently inserted at the growth cone; (*ii*) there could be a diffusible anterior-posterior biochemical gradient across each segment; (*iii*) there could be a nondiffusible biochemical gradient; for example, in the form of molecules secreted onto the basement membrane or on the surface of epidermal cells; or (*iv*) information about polarity could arise as a natural consequence of the relative positions of specific differentially labeled cells or processes within the neuropil. Whereas the first three mechanisms are temporally static, the fourth mechanism could utilize the relative timing of developmental events.

Two features appeal to us about the "labeled pathways" hypothesis. First, it is consistent with the results of ourselves and others in a wide variety of both invertebrate and vertebrate preparations. Second, it can be tested in the grasshopper embryo by lesioning specific identified axons or axon bundles and looking for a subsequent effect upon the behavior of identified growth cones.

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