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PATHFINDING BY NEURONAL GROWTH CONES IN GRASSHOPPER EMBRYOS

I. Divergent Choices Made by the Growth Cones of Sibling Neurons[1]

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Abstract

We are interested in how the growth cones of identified neurons navigate in the central nervous system of the grasshopper embryo. The behavior of identified growth cones was observed as a function of developmental time by (*i*) periodically removing embryos from synchronized clutches of eggs and (*ii*) filling identified neurons in the central nervous system with the fluorescent dye Lucifer Yellow. We examined the first six progeny of the identified neuronal precursor cell, neuroblast 7-4: the Q1, Q2, G, C, Q5, and Q6 neurons. Their growth cones extend sequentially upon the same route across the midline of the posterior commissure of the developing ganglionic neuropil. However, after reaching the contralateral side of the neuropil, their growth cones diverge from each other at reproducible, cell-specific choice points. By focusing our attention on one such choice point, at which the growth cone of the G neuron turns anteriorly while the growth cone of its sibling C neuron turns posteriorly, we hope to elucidate the environmental cues which guide individual growth cones.

The complex morphologies of neurons are largely generated very early in development during axonal outgrowth. As a growth cone advances, it leaves behind an axon or dendrite whose shape records that growth cone's history. Thus, understanding how the complex shapes of individual neurons unfold during embryogenesis requires an understanding of the choices made by their growth cones. We would like to know what environmental cues in an embryo influence the behavior of individual growth cones and how growth cones are programmed to react to those cues. Our strategy has been to examine the behavior of identified growth cones as a function of developmental time and to correlate their behavior with their mitotic ancestry and their environmental history.

The nervous system of the grasshopper embryo is an excellent preparation for studying pathfinding by individual growth cones because it is highly accessible and relatively simple (e.g., Goodman and Bate, 1981). The cell bodies, axons, and growth cones of individual neurons can be visualized *in vivo* with Nomarski interference contrast optics. They can be penetrated with microelectrodes and filled with a variety of markers. Like many other invertebrates, the grasshopper's segmentally reiterated nervous system is composed of identified neurons (e.g., Hoyle and Burrows, 1973; Burrows and Hoyle, 1973; Pearson et al., 1980; Pearson and Robertson, 1981). These identified neurons are produced by segmentally reiterated precursor cells which are themselves identifiable (Bate, 1976a; Bate and Grunewald, 1981). Thus, it is possible to construct lineages relating a neuron's order of birth from a particular precursor cell to its mature phenotype (e.g., Goodman and Spitzer, 1979; Goodman et al., 1981; Goodman and Bate, 1981; Goodman, 1982). These attributes allow us to identify and characterize the behavior of individual growth cones simply by following the morphological differentiation of the identified neurons from which they arise.

We focused our attention upon the development of the first six progeny of neuroblast 7-4 (Q1, Q2, G, C, Q5, and Q6), examining in particular the sibling G and C neurons. The growth cones of the G and C neurons travel together until they reach a stereotyped choice point in the developing neuropil. Here G's growth cone turns anteriorly, while C's turns posteriorly. Our hope is that by characterizing the cellular environment around this choice point, we will be able to elucidate the environmental cues that guide these growth cones.

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Materials and Methods

Single clusters of synchronized eggs were gathered from a laboratory colony of *Schistocerca americana*. Occasionally, we caught females in the act of laying eggs and thus knew the precise age of the embryos. Single clusters were maintained in waxed paper cups between moistened sand and cotton in a humid incubator at 33°C. The eggs hatch in 20 days under these conditions. Embryos were staged according to the method of Bentley et al. (1979) as a percentage of their total time *in ovo*. Individual eggs were removed periodically for study.

Eggs were immersed in saline, punctured at their anterior end, and cut open at their posterior end, and the embryos were squeezed gently out of the eggshell. After adhering yolk was removed and the legs were cut off, the ventral nerve cord was exposed by slitting the dorsal membrane covering the embryo from head to tail. The embryo then was transferred in a drop of saline onto a glass slide lightly coated with polymerized Sylgard 184. The embryo was positioned dorsal side up in a small rectangular coffin cut into the Sylgard and held in place by wire pins projecting from underneath the Sylgard layer and over the embryo's head and tail.

To aid visibility, the saline used in this study was slightly hypotonic and consisted of 150 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 mM TES adjusted to pH 7.0 to 7.2.

Neurons were visualized using a Zeiss compound microscope with Nomarski interference contrast optics and a Leitz × 50 water immersion lens; cell bodies and axons were impaled under direct visual control. Glass micropipettes were drawn on a Sutter Instruments puller and filled with 5 to 10% Lucifer Yellow (Stewart, 1978) dissolved in either 0.1 M LiCl or distilled water. Constant or pulsed negative currents between 0.5 and 1.5 nA filled most cells with Lucifer Yellow in about 2 min. Filled cells were visualized immediately in the living embryo by switching to blue light; cells were drawn using a Zeiss drawing tube. The cells shown in Figure 2 were fixed in buffered 10% formalin, dehydrated, and mounted in Fluormount (ICN Pharmaceuticals) before being drawn. The cells shown in Figures 8 and 9*C* were filled with HRP (Boehringer Mannheim: Grade I lyophilized) as described in the following paper (Raper et al., 1983). The cells shown in Figure 9, *A* and *B*, were filled with Lucifer Yellow and visualized using an antibody to Lucifer Yellow (anti-LY, described in detail by Taghert et al., 1982). These cells were fixed in 2% paraformaldehyde, incubated with anti-LY antibody, and then visualized using an HRP-labeled second antibody.

Background

Adult morphology of the G and C interneurons. The G and C neurons are the two cells whose morphological differentiation we have studied in the greatest detail (Fig. 1, *A* and *B*). Both neurons originally were identified and their morphologies described in the second thoracic (T2) ganglion of the adult grasshopper (Rehbein, 1976; Pearson and Goodman, 1979; Pearson et al., 1980; Pearson and Robertson, 1981). There is a pair of bilaterally symmetric G neurons and a pair of bilaterally symmetric C neurons within the T2 ganglion. The major neurite of each G neuron extends across the ganglionic midline and then runs anteriorly in the lateral portion of the contralateral connective (ventral nerve cord). A smaller neurite branches from the major axon and extends posteriorly near the center of the contralateral connective and into the T3 ganglion. The G neuron's "omega-shaped" dendrites project anteriorly near the midline of the T2 neuropil. The morphology of the C neuron shares some of the features of the G neuron, yet possesses striking differences as well. The major neurite of each C neuron also crosses the ganglionic midline. However, rather than continuing anteriorly like G's, C's major neurite extends posteriorly near the lateral margin of the contralateral connective. The C neuron's "antler-shaped" dendrites also extend anteriorly in the T2 neuropil but are medial to G's dendrites.

Synaptic connections. Some of the synaptic inputs and outputs of the G and C neurons have been described in the adult (Pearson et al., 1980; Pearson and Robertson, 1981) and are summarized in Figure 1*C*. Both are depolarized by the descending contralateral movement detector interneuron. Both excite the fast extensor tibia motor neuron on the contralateral side of the next posterior (T3) ganglion. Not all of the synaptic connections made by the G and C neurons are the same. The G neuron makes an excitatory synaptic connection onto the C neuron (K. G. Pearson and R. M. Robertson, personal communication). This connection probably is mediated by the medially directed process which branches from G's major ascending axon in T2. The C neuron excites a group of flexor tibia motor neurons in T3, while the G neuron excites the M interneuron which in turn inhibits the same flexor motor neurons.

G's and C's differing branching patterns in T3 reflect their innervation of overlapping but different targets and their differing functions. Pearson and Robertson (1981) hypothesize that by exciting both the extensor and flexor motor neurons (Fig. 1*C*), C evokes the initial phase of the escape jump called the co-contraction by Heitler and Burrows (1977a, b). G is thought by Pearson et al. (1980) to be one of many inputs onto the M neuron which in turn serves to trigger the actual jump by inhibiting the flexor motor neurons.

Results

Ultimately, we would like to explain how G and C assume their distinctive morphologies and locate their appropriate targets by explaining how their growth cones extend, turn, and branch at specific places in the developing neuropil. This is only possible by studying their differentiation at very early stages of development, since by 70% of embryogenesis they have assumed most of their adult morphological characteristics (Fig. 2). By 70%, the G neuron's major axon extends anteriorly, its smaller axon extends posteriorly, its dendrites have their normal "omega-shaped" appearance, and a medially directed collateral extends from the major anteriorly extending axon (Fig. 2*A*). The C neuron's major axon descends posteriorly and branches in the next posterior segment in characteristic locations. At the same time, its major dendrites are beginning to assume their "antler-shaped" appearance (Fig. 2*B*).

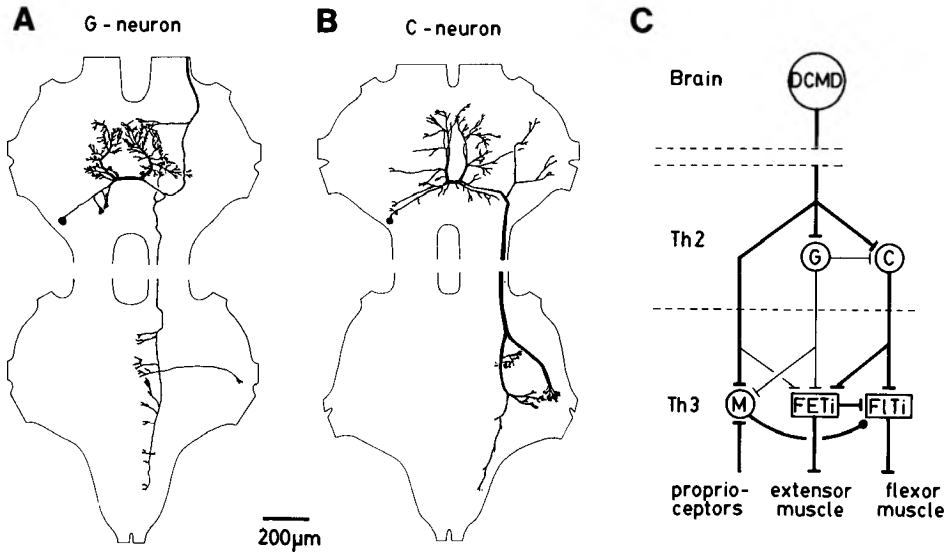

Figure 1. Adult characteristis of the G and C interneurons. The adult morphologies of (*A*) the G neuron and (*B*) the C neuron. Camera lucida tracings were made of separate fixed whole mount preparations in which each cell's identity was established by physiological criteria and then filled with Lucifer Yellow. *C*, Synaptic interactions involving the G and C neurons in the adult. Excitatory connections are indicated by *bars* and inhibitory connections are indicated by *solid circles*. *DCMD*, descending contralateral movement detector interneuron; *FETi*, fast extensor tibia motor neuron; *FlTi*, flexor tibia motor neuron. (Figure courtesy of K. Pearson and based upon the data of Pearson et al., 1980; and Pearson and Robertson, 1981).

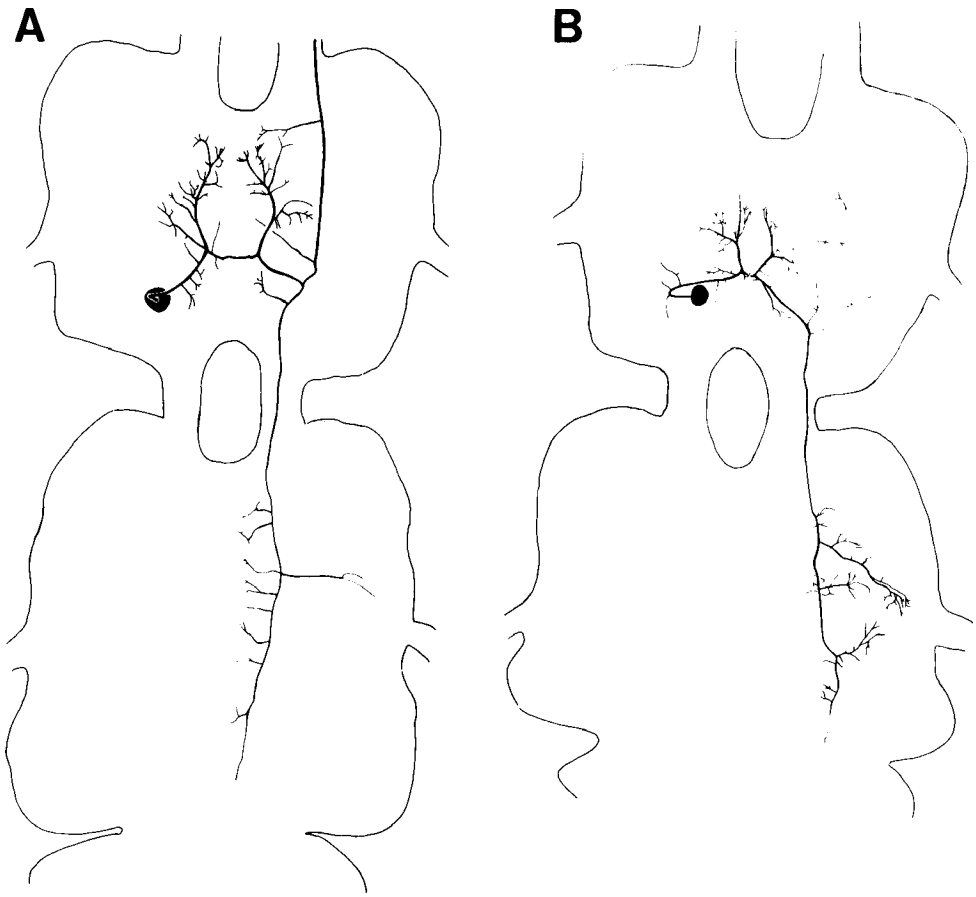

Figure 2. Morphologies of the G and C interneurons at 70% of embryogenesis. Camera lucida tracings were made of neurons filled with Lucifer Yellow from fixed whole mount embryos. *A*, The G neuron; *B*, the C neuron.

Lineage of G and C neurons. Each segmental ganglion in the grasshopper is generated by a precise segmentally repeated pattern of precursor cells. The neurons in each segment are generated by two bilaterally symmetric plates containing 30 neuroblasts (NBs) each and an additional unpaired median neuroblast (Bate, 1976a), and seven midline precursor cells (Bate and Grunewald, 1981). Each neuroblast is a stem cell, maintaining its

large size as it divides repeatedly to produce a chain of smaller ganglion mother cells (Bate, 1976a). Each ganglion mother cell in the chain divides once more, thus producing a chain of paired ganglion cells which subsequently differentiate into neurons. The two bilaterally symmetric neuroblasts NB 7-4 (Fig. 3*A*) each push a string of progeny up toward the dorsal posterior surface of every segment. Because of their location on the dorsal surface of the ganglion, the progeny of NB 7-4 can be visualized easily and are highly accessible to microelectrode penetration throughout their development.

We have used three techniques to relate the birth order of individual neurons from NB 7-4 to their neuronal identity. First, we reconstructed the lineage by observing the living embryo with Nomarski optics and filling the cells with Lucifer Yellow. Unfortunately, we are not able to reconstruct lineages by actually watching a single embryo develop (as is done in the nematode; e.g., Sulston and Horvitz, 1977), but rather by (*i*) examining the same segment in embryos at intervals of several hours (less than 1% of development) from synchronized clutches of eggs and by (*ii*) examining different segments in the same embryo (there is a gradient of development across the 3 thoracic and 11 abdominal segments of less than 1%/segment). Second, we have confirmed this lineage by observing the NB 7-4 family in reconstructions of Michael Bate's photo album of serial 1-μm plastic sections, taken at intervals of time corresponding to about 1% of development (C. M. Bate and C. S. Goodman, manuscript in preparation). Third, we have confirmed this lineage by injecting the cells with Lucifer Yellow and observing the pattern of dye coupling through the chain of progeny backwards to NB 7-4 (Goodman and Spitzer (1979) showed that NBs are dye coupled to their progeny until the time of axonal outgrowth).

The first ganglion mother cell born from NB 7-4 divides and gives rise to a pair of identified neurons named Q1 and Q2 (Fig. 3*B*). The second ganglion mother cell gives rise to a pair of neurons which differentiates into the G and C neurons described earlier. The third ganglion mother cell gives rise to a pair of identified neurons named Q5 and Q6.

Order of axonal outgrowth. The Q1 cell is the first of the NB 7-4 progeny to initiate axonal outgrowth (Fig. 4*A*). Q1's growth cone is among the first growth cones to cross in the posterior commissure and pioneers the pathway which its later NB 7-4 siblings follow. Q1's growth cone extends at about 10 to 15 μm/hr toward the ganglionic midline. There it meets the axon of its contralateral homologue and extends upon it into the contralateral neuropil. The growth cone of Q2 follows several hours and about 50 μm behind the growth cone of Q1. Many filopodia emerge from the Q2 cell body before its axonal process is evident. After crossing the midline, Q1 and Q2 turn posteriorly at a characteristic position onto a stereotypic medial longitudinal pathway (Fig. 4*B*). Q1 and Q2 appear to die shortly thereafter, since two clumps of condensed cellular debris occupy what used to be the positions of their cell bodies (they sometimes die one at a time, with one living cell and one clump of debris observed in their characteristic location). Subsequent to this period of cell death, we have failed to locate the Q1 and Q2 cells in exhaustive searches in which all surrounding cells were filled one at a time with Lucifer Yellow.

The G cell is the third progeny of NB 7-4 to initiate an axonal process. It extends across the ganglionic midline upon the pathway in the posterior commissure pioneered by Q1 and followed previously by Q2 (Fig. 4*B*). A few hours later, C initiates an axon which follows behind G's. The next two progeny of NB 7-4, Q5 and then Q6, subsequently follow C across the ganglionic midline upon the same pathway (Fig. 4*C*). It is important to note that even by the time Q2 enters the posterior commissure there are already several other axonal bundles in the commissure, yet Q2's growth cone and those of the next four progeny of NB 7-4 (G, C, Q5, and Q6) all remain in the axon bundle pioneered by Q1. The divergent choices of the growth cones of Q1, Q2, G, C, Q5, and Q6 are summarized in Figure 3*C*.

The cell bodies of the G and C neurons can be located individually without having to fill them with dye. Throughout development they sit in a characteristic location upon the dorsal surface at the posterior end of the ganglion, just lateral to the longitudinal axonal path-

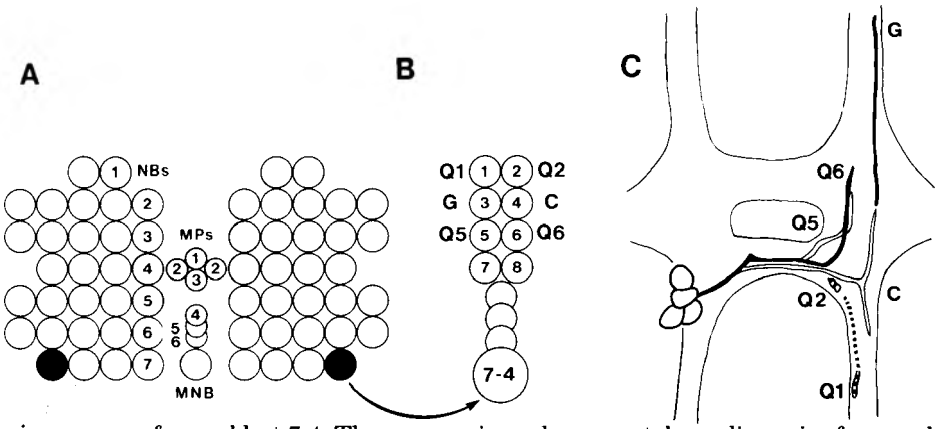

Figure 3. The first six progeny of neuroblast 7-4. The neurons in each segmental ganglion arise from a plate of 61 neuroblasts (NBs) and 7 midline precursor (MPs) cells. *A*, NB 7-4 (*solid circles*) is the posterior, lateralmost neuroblast on each side of every segment. *B*, The first ganglion mother cell (see the text) born from NB 7-4 divides once and gives rise to the Q1 and Q2 neurons. The second ganglion mother cell gives rise to the G and C neurons. The third ganglion mother cell gives rise to the Q5 and Q6 neurons. *C*, The axons of all six siblings cross the ganglionic midline in the posterior commissure but diverge from each other at specific locations in the contralateral neuropil (see the text). Compiled from two superimposed camera lucida tracings of Lucifer Yellow-filled neurons in living preparations before and after the deaths of Q1 and Q2.

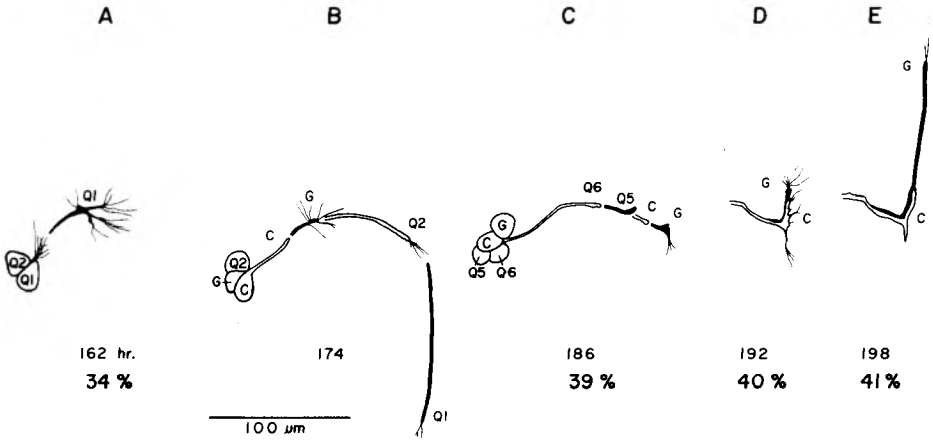

Figure 4. Order of axon initiation from the early progeny of NB 7-4. The data in this figure and Figures 6 and 7 were obtained by sampling eggs from the same synchronized egg cluster. *A*, Q1 (*darkened axon*) is the first of 7-4's progeny to initiate an axonal process. Many fine filopodia extend from Q2 at its site of axonogenesis. *B*, After 12 hr, Q2 (*open axon*) has followed Q1 across the ganglionic midline and posteriorly in the medial portion of the connective. First G (*darkened axon*) and then C (*open axon*) have initiated axonal processes which follow Q1 and Q2 across the midline. *C*, Twelve hours later, G leads C to the lateralmost portion of the contralateral neuropil. Q5 (*darkened axon*) and Q6 (*open axon*) have initiated axonal processes. *D*, Six hours later, C's growth cone has caught up to G's. Both growth cones extend for short distances in an anterior-posterior direction at the lateral edge of the neuropil. *E*, After an additional 6 hr, G has begun extending anteriorly at a rapid pace.

ways and just posterior to the posterior commissure. Throughout development they usually are larger than any of their immediate neighbors. At the time when G's growth cone has made the anterior choice which distinguishes it from C, G's cell body is either anterior or lateral to C's (e.g., Fig. 4). The first of the pair to initiate axonogenesis is anterior or lateral to the other, and we therefore conclude that G is the first to extend an axonal process (Fig. 5).

The growth cones of first G and then C extend past the location in the contralateral neuropil where Q1 and Q2 turned posteriorly (see Fig. 3*C* and Raper et al., 1983). G's growth cone continues laterally until it reaches a specific location at the lateral margin of the contralateral neuorpil. Here it often appears to pause for periods of up to 10 hr. For example, in the egg cluster used to make Figure 5, G's growth cone remained at approximately the same location for 7 hr. During this period in which G's growth cone stops extending laterally, C's growth cone catches up to G's. At the same time, the distalmost portion of G's growth cone often appears to creep slowly anteriorly. It is quite common for the G and C growth cones to have nearly identical morphologies and positions in the neuropil for many hours (Figs. 4 and 5). During this period in which the two growth cones are superimposable, we gained the impression from simultaneous Lucifer Yellow fills that G's growth cone was generally dorsal to C's. In reconstructions of the G and C growth cones using semi-serial electron micrographs, we found that at the location where G turns anteriorly, C was dorsal to G in three of five preparations (Raper et al., 1983; M. Bastiani, J. A. Raper, and C. S. Goodman, manuscript in preparation). At the anterior tip of C's growth cone, G was dorsal to C in four of the same five preparations.

The growth cones of G and C diverge at a specific choice point. Once G's growth cone has begun to extend

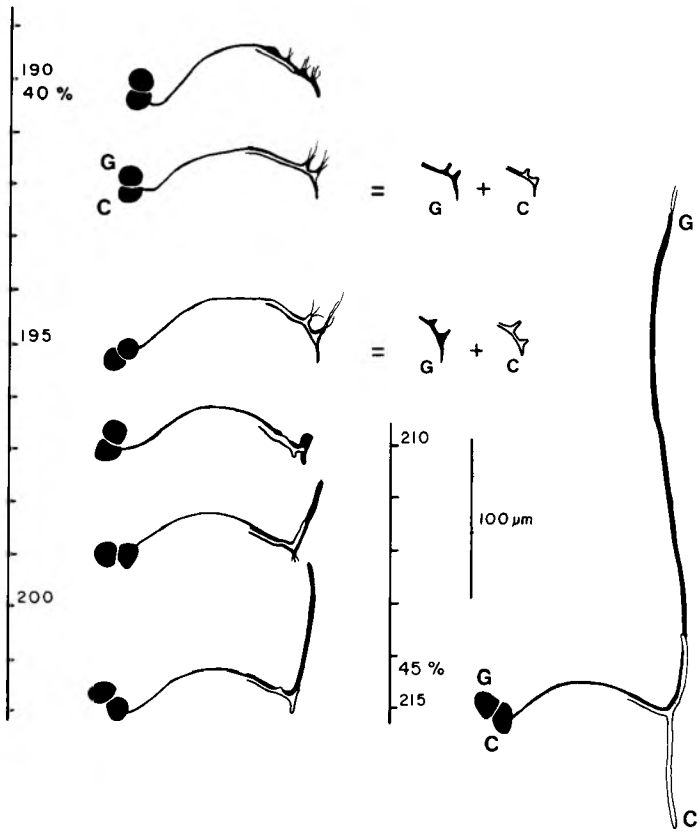

Figure 5. The morphologies of the G and C growth cones at their point of divergence. G (*darkened axon*) leads C (*open axon*) across the ganglionic midline. At a distal location in the contralateral neuropil, G's extension slows, and C's growth cone catches up to G's. For a brief period their growth cones assume nearly identical positions in the neuropil and have very similar morphologies. The G and C growth cones are shown in their natural relationship (superimposed) and separately. G then extends anteriorly, leaving C behind.

anteriorly, its rate of growth increases to about 20 μm/hr. This period of rapid elongation leaves C's growth cone behind at the choice point. C's growth cone branches and extends quite slowly either anteriorly (e.g, Fig. 4*D* and 5 at 199 hr) or symmetrically in both the anterior and posterior directions (e.g., Figs. 4*E* and 6*A*) for between 6 and 18 hr. Its posteriorly directed branch eventually begins to elongate rapidly into the third thoracic (T3) ganglion and beyond (Fig. 6, *B* and *C*). After C's major axon has passed through T3, many fine processes extend both medially and laterally in the T3 neuropil (Fig. 6*D*). Within a short time, those lateral branches that correspond to the locations of lateral neurites in the adult extend in their characteristic directions (Fig. 6*E*). Within 24 hr these neurites have thickened and are continuing to advance toward the neurites of the flexor and extensor motor neurons (see "Background"), while many fine branches are no longer evident (Fig. 6*F*).

G assumes its mature morphology in a direct manner. At around 50% of embryogenesis its major neurite has already crossed the ganglionic midline and ascended through several segments in the lateral margin of the ganglionic connective (Fig. 7*A*). A fine process branches from G's major neurite and descends in the contralateral connective for a short distance. This fine process later advances into T3 (Fig. 7*B*). By the time its growth cone reaches the posterior commissure of T3, a laterally directed process emerges from it in the position (Fig. 7*C*) where G's major output branch is found in T3 of the adult. At the same time, a medially directed process has

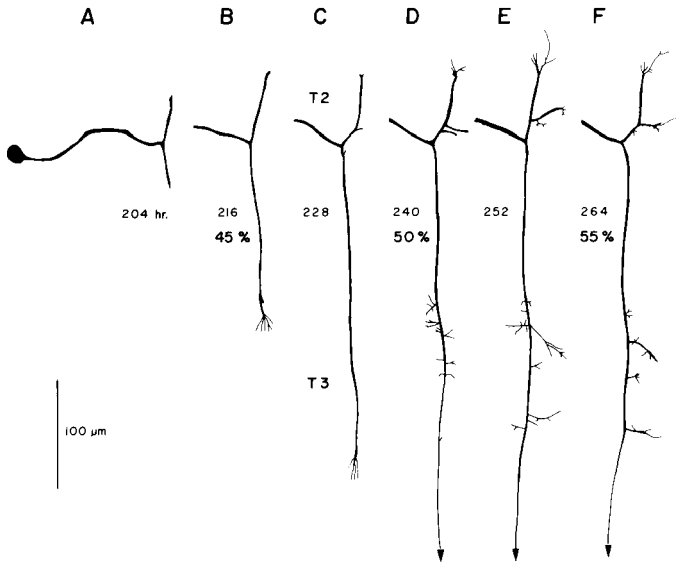

Figure 6. Further development of the C neuron. The data in this figure represent futher samples at 12-hr intervals from the same synchronized clutch as in Figure 4. *A*, C's growth cone extends slowly in both the anterior and posterior directions for many hours after G has begun its rapid anteriorward extension. *B* and *C*, The posterior branch of C's growth cone continues to elongate, while the anterior branch does not. *D*, The anterior branch sprouts a few thick collaterals, while the posterior branch has many very thin collaterals. *E* and *F*, Collaterals characteristic of C's mature morphology in both the T2 and T3 segments extend toward their appropriate locations in the neuropil.

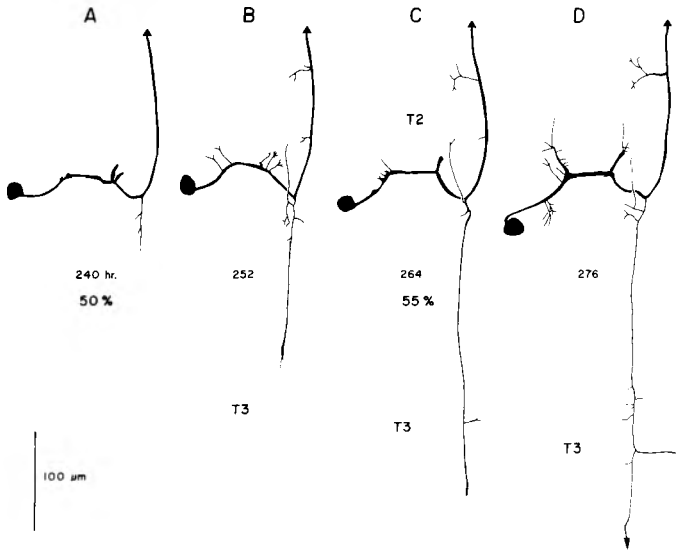

Figure 7. Further development of the G neuron. The data in this figure represent samples at 12-hr intervals from the same synchronized clutch as in Figures 4 and 6. *A*, G's major axon extends anteriorly through several segments, while a much thinner process has just begun to advance posteriorly. *B*, Twelve hours later, this thin, posteriorly directed axon has just entered the T3 ganglion. Medially directed collaterals have sprouted from G's major neurite in T2. *C*, After an additional 12 hr, the more anterior of these collaterals in T2 has increased in its extent. The thin, posteriorly directed axon has advanced through the T3 segment and a laterally directed collateral corresponding in position to a prominant neurite in the adult has appeared. *D*, Twelve hours later, several additional medially directed neurites are evident in T3. In T2, G's dendrites have begun to grow anteriorly.

branched from G's major neurite in the anterior portion of the T2 neuropil. At about this same developmental time, G's dendrites begin to elongate in T2.

Complexity of growth cones. We observed the filopodia of growth cones by visualizing cells with either HRP (Figs. 8 and 9*C*) or an antibody to Lucifer Yellow (Taghert et al., 1982) (Fig. 9, *A* and *B*). The shape of the G growth cone and its filopodia depend upon its location in the neuropil (Fig. 9). As the G growth cone reaches its choice point and slows down, it usually becomes quite broad and complex in shape (about 20 μm long and 5 μm wide). It sometimes has several anteriorly directed bumps a few micrometers in length at reproducible locations proximal to its distalmost tip. Filopodia extend in tufts from the bumps or from the positions in which bumps are likely to occur. We call these locations "active sites" because (*i*) they are locations of active filopodial extensions and (*ii*) they appear to be the sites from which growth cone extension occurs.

The G growth cone becomes long and tapered once its rapid growth into the connective has begun. It sometimes has one or more terminal processes extending in its direction of growth which are less than 1 μm in diameter but which are thicker than filopodia (typically 0.1 to 0.2 μm in diameter). These processes have been called "terminal filaments" by Shankland (1981). The terminal filaments break up into numerous filopodia at the ad-

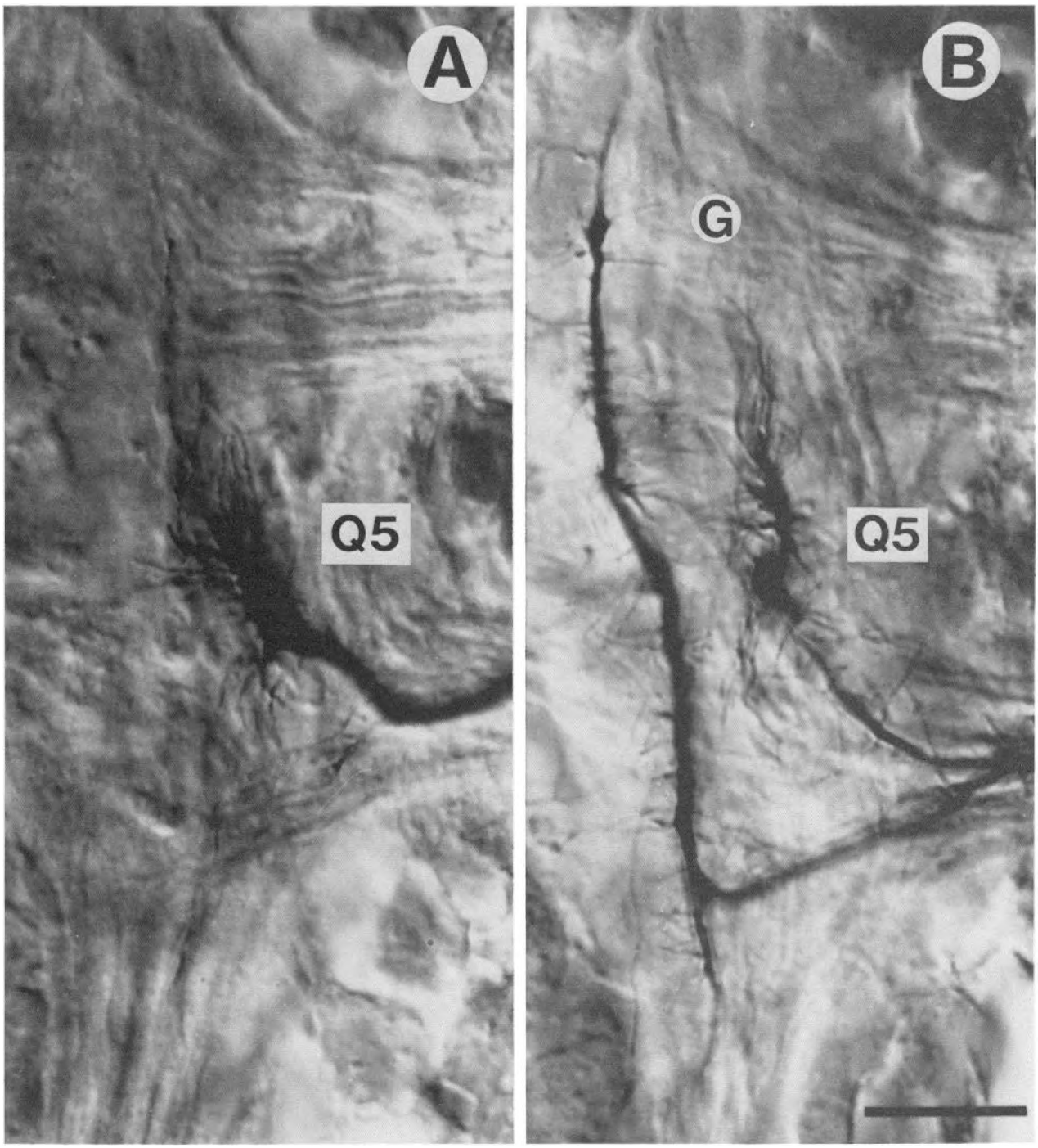

Figure 8. Photographs of identified growth cones. Whole mount preparations of embryonic neurons filled with HRP. *A*, The growth cone of a Q5 neuron just as it is beginning to turn anteriorly in the medial portion of the neuropil. The Q5 cell body is not shown and is to the right. *B*, The growth cones of G and Q5 slightly later in development. Note that G's growth cone turns anteriorly in a much more lateral portion of the neuropil than Q5. Both the G and Q5 cell bodies are not shown and are to the right.

vancing tip. In other cases, there are no obvious terminal filaments, and filopodia simply extend in profusion from the leading edge of the tapered growth cone. We do not feel that these differing shapes represent dichotomous classes of growth cones, but rather variations in the extent of growth cone tapering. In either case, the terminal filopodia are often very long (up to 100 μm or more) and generally extend anteriorly along the route the growth cone will follow. Other shorter filopodia extend laterally from the growth cone. The young axon behind the growth cone generally has numerous lateral filopodia that later disappear, as described for other neurons in grasshopper embryos (e.g., Goodman and Spitzer, 1979; Goodman and Bate, 1981).

Variability in the relative positions of identified growth cones. We do not want the time lines presented

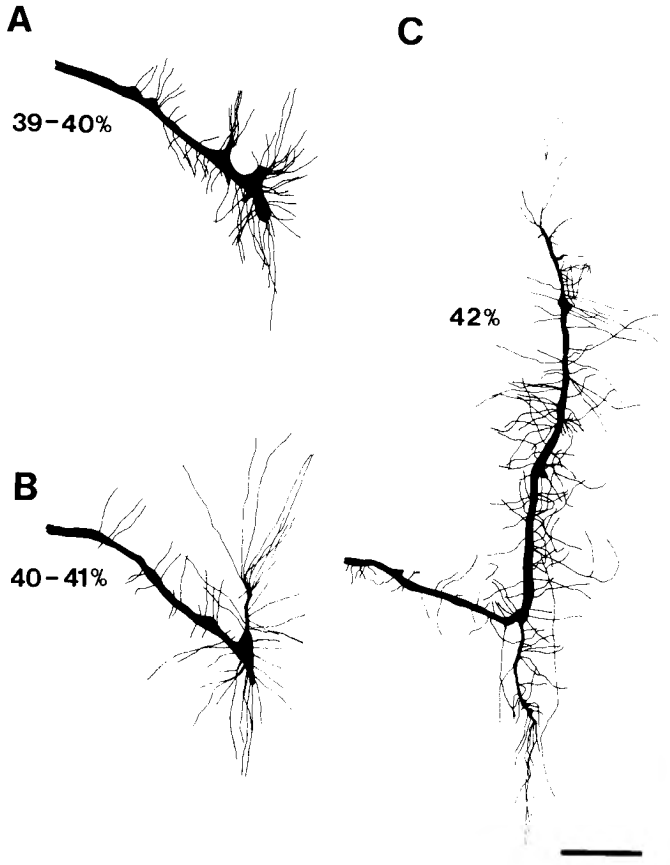

Figure 9. Fine structure of the G growth cone. *A*, The morphology of the G growth cone before it turns anteriorly is large and complex. Filopodia radiate in profuse tufts from reproducible locations called active sites (see the text) at which "bumps" generally are observed. *B*, Just as G begins to extend anteriorly, many long filopodia project along or near the path G's growth cone will follow. *C*, As G proceeds anteriorly, filopodia continue to project along the path it will take. The growth cone is sometimes tapered, and many lateral filopodia always extend from the newly formed axon behind it. *A* and *B* are camera lucida tracings of neurons filled with Lucifer Yellow and visualized with an anti-Lucifer Yellow antibody reacted with an HRP-conjugated second antibody. The cell shown in *C* was filled directly with HRP. *Scale bar*: 20 μm.

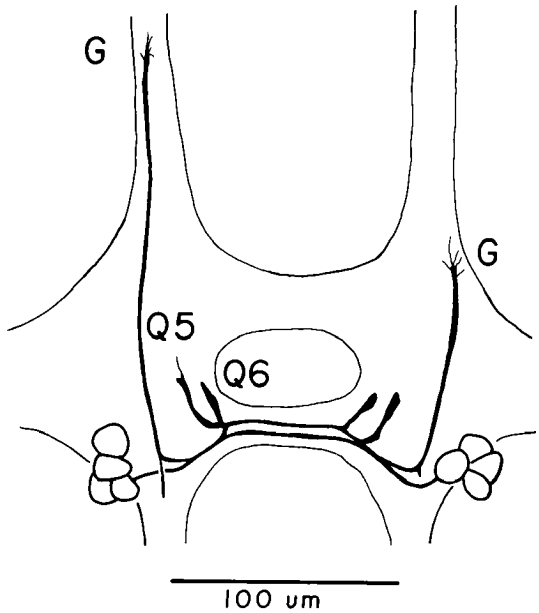

Figure 10. Variability in the location of the G growth cone. An approximately 42% embryo in which the growth cones of the bilaterally symmetric G cells extend for different distances in the ganglionic connectives.

in this paper to leave the mistaken impression that a given growth cone is always found in a precise position at a particular developmental time. For example, when we examine the locations of both G growth cones in a single segment, we sometimes find that they have grown to different lengths (either side can be longer). Figure 10 shows an extreme example of this variability. G's growth cone on one side has advanced about 100 μm further anteriorly than that of its contralateral homologue. Note that the growth cones of Q5 and Q6 are in comparable locations on both sides of the same segment, suggesting that the G axons are not different lengths simply because one side of the ganglion is more mature than the other. Similar variability in a single segment was observed for the growth cones of the other cells examined (C, Q5, and Q6) and also was observed when we examined the same growth cone in different embryos of the same age. This variability in the relative positions of growth cones indicates that some variability occurs in the absolute timing of some developmental events but could still be consistent with a highly invariant ordering of those same events.

Variability in the morphology of the G cell. We have found a few cases in which G's ascending axon appears to extend anteriorly from an unusual position in the neuropil. In Figure 11*A* is shown the morphology of a G axon which extends anteriorly from the "normal" distal position in the contralateral neuropil. The G axon in Figure 11*B* appears to have jumped onto its characteristic ascending pathway from a slightly more medial position in the contralateral neuropil. In a minority of the egg clusters we examined, this configuration was the most common morphology of the G cell. In one preparation (Fig 11*C*), we found that the G and C cells jumped upon their characteristic pathway from an even more medial position than in Figure 11*B*.

These anomalous morphologies of G could arise if active sites proximal to the distal tip of G's growth cone are able to recognize and extend upon G's ascending pathway. Alternatively, they could arise if the distal tip of G's growth cone, without ever growing to its normal lateral position, occasionally jumps onto G's ascending pathway earlier than usual in development. This second alternative is unattractive since in some embryos, the distal tip of G's growth cone had reached its normal lateral position in the contralateral neuropil, while a second more medial branch of G's growth cone had jumped independently onto G's ascending pathway and already was growing anteriorly. Thus, it is likely that each active site on G's complex growth cone is capable of recognizing and extending anteriorly upon the lateral pathway G follows in the connective.

Discussion

The G and C neurons acquire their specific morphological characteristics in a highly ordered and stereotypic

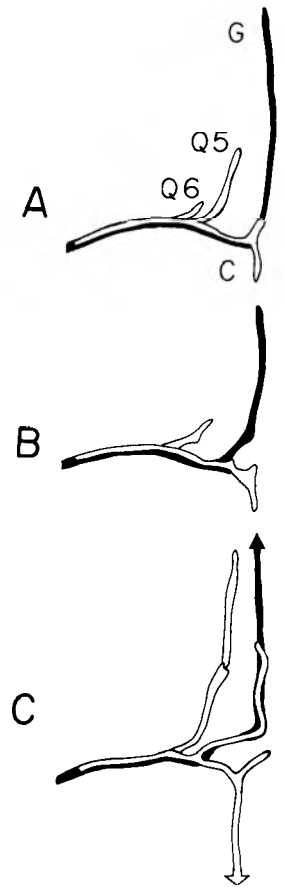

Figure 11. Variability in the morphology of the G cell at its first choice point. *A*, Most G cells extend anteriorly from a distal position in the contralateral neuropil. *B*, In rare clutches it was common for G cells to extend from a slightly more medial position in the neuropil corresponding to a bump just medial to the distalmost tip of the C neuron's process. *C*, In one instance the G neuron and the anterior branch of the C neuron extended anteriorly from a highly medial position.

manner during development. Their major growth cones extend directly to specific locations in the neuropil where they turn or branch onto very precise pathways. The primary processes thus laid down sprout collaterals at specific, reproducible points which correspond to the locations of particular branches in mature G and C neurons. These observations discount the possibility that these neurons assume their characteristic adult morphologies by a process of random growth followed by the selective pruning and survival of only appropriate neurites. Instead, the picture emerges of a tightly choreographed pattern of linear extensions of growth cones from choice point to choice point, with the concomitant requirement that growth cones navigate with precision in the developing neuropil.

Studies in developing vertebrate nervous systems show that growth cones must actively differentiate between specific guidance cues. One of the simplest ways in which growth cones could navigate is by passively following mechanical guidance cues. Anatomical studies in amphibians have revealed channels between ependymal cells that are subsequently invaded by growth cones (Singer et al., 1979; Silver and Robb, 1979; Silver and Sidman, 1980). These observations have given rise to the "blueprint" hypothesis, whereby the germinal neuroepithelium is thought to contain the pattern for channels which in turn serve as mechanical guides for elongating axons. However, even this hypothesis depends upon the presence of specific biochemical cues which are actively sought by elongating axons during pathway selection. For example, Singer et al. (1979) write:

> "In addition to providing specific highways . . . the blueprint hypothesis implies that individual axons 'recognize' and follow particular itineraries even when challenged by multiple highways."

The experiments of Lance-Jones and Landmesser (1980a, b; 1981a, b) demonstrate that some form of specific guidance cues is sought actively by the growth cones of motor neurons innervating the chick hindlimb. At no time during normal development do they find evidence of a widespread or random distribution of innervating axons within the limb (Lance-Jones and Landmesser, 1981a). From their observations they exclude models in which random axonal outgrowth is corrected later by cell death and/or axon retraction, or models in which axons maintain a fixed topographic position with respect to each other. After rotating small sections of spinal cord, Lance-Jones and Landmesser (1980b, 1981b) found that motor neurons consistently innervate their correct targets in spite of the noval axonal trajectories by which their axons enter the nerve plexus at the base of the limb bud. They conclude that motor neuron growth cones actively seek out specific guidance cues, which are not global in nature but rather are likely to be localized in the region where the plexus forms.

These findings in vertebrate nervous systems parallel our observations in the grasshopper embryo. It is highly unlikely that the growth cones we have been studying are directed passively by mechanical guidance cues. NB 7-4's early progeny enter the contralateral neuropil at the same location but then diverge from each other. The growth cones of G and C are found to be in nearly identical positions just before they elongate in opposite directions. Nor do neurons acquire their characteristic morphologies by growing processes in random directions and then later withdrawing those which are inappropriate. Single identified growth cones are observed to navigate with great precision upon reproducible, cell-specific routes in the developing neuropil. The important question, then, is how do individual growth cones locate, recognize, and react to particular locations in the neuropil?

The first implication of these findings is that there are either structural or biochemical heterogeneities in the neuropil which correlate with growth cone behavior. These heterogeneities may be in the form of global gradients (e.g., Sperry, 1963; Trisler et al., 1981). Alternatively, they could be in the form of marked pathways, perhaps analogous to the linearly arranged guidance cues which Katz and Lasek (1979, 1980, 1981) call "substrate pathways," or they could be discrete landmarks which serve as "stepping stones" for specific growth cones. This model has been proposed as one of the mechanisms involved in the pioneering of peripheral (Bate, 1976b; Ho and Goodman, 1982) and central (Goodman et al., 1982;

Taghert et al., 1982) axonal pathways in the grasshopper. In a companion paper (Raper et al., 1983) we suggest that the growth cones of the G and C neurons are programmed to elongate upon the axons of identified neurons which differentiated earlier in the ganglionic neuropil.

The second implication of the precision with which growth cones extend and turn is that particular growth cones are determined to react in nonrandom, characteristic ways to whatever heterogeneities exist in the neuropil. Several observations suggest that different growth cones are programmed to react differently to the same environmental cues. For example, the growth cones of the G and C neurons (*i*) pass by the location in the neuropil where the growth cones of Q1 and Q2 have turned posteriorly, (*ii*) pass by the location where the Q5 and Q6 growth cones will turn anteriorly, and (*iii*) occupy nearly identical positions in the neuropil before they diverge in opposite directions.

Alternatively, the growth cones of the early progeny of NB 7-4 may have identical programs but may behave differently as they pass through a given location because (*i*) they reach it at different times and (*ii*) the location's properties change over time. This could explain how the G and C growth cones pass by the location where Q1 and Q2 previously turned posteriorly. It would be much more difficult, however, to explain how G and C extend in opposite directions after their growth cones simultaneously occupy nearly identical positions in the neuropil. Here one would have to invoke competitive interactions between the G and C growth cones or propose that their divergent behavior depended upon the 3-hr difference in their arrival time at the choice point. Another explanation is that identically programmed growth cones could be exquisitely sensitive to even very small differences in their relative locations. Arguing against this alternative explanation, however, is our failure to detect an invariant orientation of the G and C growth cones even within the axon bundle upon which they diverge (M. Bastiani, J. A. Raper, and C. S. Goodman, manuscript in preparation).

These considerations are related to another question of fundamental importance. NB 7-4's early progeny all cross the ganglionic midline before they turn onto specific pathways in the contralateral neuropil. What prevents them from turning onto homologous pathways in the ipsilateral neuropil before they have reached the midline? The axial symmetry of the grasshopper segmental nervous system makes the hypothesis that sidedness is marked unattractive to us. Experimental manipulations in insects have shown that regenerating motor and sensory neurons will innervate the side opposite from the side they would normally innervate (Bate, 1976c; Palka and Schubiger, 1975). Experimental manipulations in vertebrates have shown that a Mauthner axon can extend in the spinal cord on the side opposite from that in which it is normally found (Stefanelli, 1951; Hibbard, 1965). Perhaps a given pathway expresses the characteristics which distinguish it from other pathways only after the growth cones which are determined to follow it have already crossed the midline, or growth cones which are determined to follow a given pathway may not be able to recognize or respond to that pathway until they are sufficiently mature or until they have experienced another event (e.g., crossing the midline) which primes them for their next task.

We have been impressed by the complexity of the G and C growth cones at their point of divergence. Tufts of filopodia radiate from specific "active sites" characteristic of particular developmental times. Filopodia can radiate away from each active site for up to 50 μm, allowing it to sample a large portion of the territory surrounding the growth cone. The variations we have seen in G's morphology at later times (Fig. 11) suggest that each active site may be capable of recognizing the pathway that the growth cone as a whole is determined to follow. Thus, independent active sites may compete for both the extracellular signals and intracellular resources necessary for growth cone extension. This hypothesis could be tested by observing the responses of living growth cones to manipulations in their environment.

There is no reason to assume that the fundamental mechanisms which guide a given growth cone are different at one choice point as opposed to another. For that reason we have focused our attention on the earliest cell-specific choices made by the G and C growth cones since they are made when their environment, the developing neuropil, is relatively simple. In this way we hope to be able to describe completely their cellular environment and then manipulate that environment in a precise way. In the next paper (Raper et al., 1983) we characterize the cellular environment at the choice point where the G and C growth cones diverge and suggest that G and C are differentially determined to recognize and extend upon specific identified axons.

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