Pioneer Growth Cone Steering Along a Series of Neuronal and Non-Neuronal Cues of Different Affinities

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We have analyzed the morphology of over 5000 T11 pioneer growth cones labeled with anti-HRP, which reveals the disposition of axons, growth cone branches, and filopodia. T11 axon pathways typically consist of a sequence of 7 characteristically oriented segments, with a single, distinct reorientation point between each segment. Growth cones exhibit the same orientations and reorientations in a given region as do axon segments at later stages. The single, distinct reorientations suggest that growth cones make discrete switches between guidance cues as they grow.

T11 growth cones are guided by various types of cues. A set of 3 immature identified neurons serves as nonadjacent guidepost cells and lies at the proximal end of 3 of the axon segments. To form another segment, growth cones reorient along a limb segment boundary within the epithelium. Growth cones also respond consistently to, and orient toward, a specific mesodermal cell, which may be a muscle pioneer. Thus, growth cones respond to at least 3 different types of cells in the leg.

T11 growth cones exhibit a hierarchy of affinity for these cues. Guidepost neurons are the dominant cues in that contact with them reorients growth cones from guidance by the other types of cues.

Growth cone branches are exclusively oriented to specific cues. Growth cones reorient by extending a branch directly to the cue of highest affinity and by withdrawing any branches that are extended to a cue of lesser affinity. A single filopodium in direct contact with a guidepost neuron can reorient a growth cone that still has multiple filopodia or even prominent branches specifically oriented to a previous cue of lesser affinity. These observations suggest that growth cone steering may not result simply from passive adhesion and filopodial traction, but may involve more active processes.

During the course of development, axons grow along specific pathways to their appropriate targets. Many axons are guided by fasciculating with previously formed axons and nerves. However, the initial axons growing through any region must "pioneer" a pathway through an axonless environment, and therefore must be guided by other types of cues. We are investigating the guidance mechanisms for afferent pioneer axons in the legs of grasshopper embryos.

Bate (1976) identified the first peripheral axons to arise in embryonic legs and antennae of grasshoppers as those of pairs of neurons located at the tips of those appendages. In legs, these

(T11) pioneer axons extend proximally from their cell bodies, and then turn and grow circumferentially before making a second turn and continuing proximally to the CNS (Bentley and Keshishian, 1982a, b; Ho and Goodman, 1982).

Along their route, the T11 axons extend between a set of putative guidepost neurons, with which they selectively dye-couple. Of these neurons, the T11 neuron and (pair of) Cx1 neurons are located at the characteristic turns on the route (Bentley and Keshishian, 1982a, b; Ho and Goodman, 1982; Keshishian and Bentley, 1983a-c). Selective ablation of the Cx1 guidepost cells has shown them to be necessary for normal pathfinding (Bentley and Caudy, 1983a, b). Another cue for T11 growth cones is a limb segment boundary, which orients them circumferentially (Bentley and Caudy, 1983b; Caudy and Bentley, 1986). There also is evidence that a proximal increase in epithelial affinity within segments can guide T11 growth cones proximally when they are not in contact with higher affinity cues (Caudy and Bentley, 1986).

Here we describe the detailed route taken by the T11 growth cones in normal embryos, and their characteristic interactions with identified guidepost neurons and other extrinsic cues. These interactions, and the detailed dispositions of growth cone branches and filopodia, suggest that the T11 growth cones have different affinities for their different cues. These interactions and dispositions also are interpreted with regard to their implications for models of growth cone steering based on adhesion-mediated (Bray 1979, 1982; Letourneau, 1975a, b, 1979, 1983); or active (Cooper and Schliwa, 1985) responses to guidance cues.

Materials and Methods

Antibody labeling

The anti-HRP labeling protocol is described in detail in Caudy and Bentley (1986). In brief, it consists of the following steps: (1) fixation in 4% formaldehyde in physiological saline; (2) 1 hr rinse in PBS with Triton ("rinse"); (3) 12-24 hr incubation in 0.02% rabbit anti-HRP serum; (4) 1 hr wash in rinse with 1% BSA; (5) 12-24 hr incubation in 0.04% TRITC-conjugated goat anti-HRP (IgG fraction); (6) 1 hr wash in rinse with 1% BSA; and (7) whole-mount under coverglass with 40 μ m wire spacers between coverglass and slides in 1:9 PBS:glycerol mountant with antioxidants. Using this labeling technique, we have examined the morphologies of approximately 5000 T11 growth cones (2 T11s/leg) in approximately 500 animals from about 50 different clutches of eggs.

Terms

Filopodia usually can be clearly distinguished from branches. Filopodia are the smallest diameter processes observed ($<0.5 \mu$ m) and typically are very uniform in diameter from their tip to within 2 or 3 μ m of their base. *Filopodial clusters* are several filopodia extended in a highly oriented group with few or no filopodia extended within the adjacent regions. *Branches* of growth cones are any processes larger than a filopodium or smaller than an axon. They can vary widely in diameter—from 0.5 to about 5 μ m—and also can form branches of their own.

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Table 1. Mean length of, and guidance cues for, the characteristic Ti1 axon segments^a

Segment		Mean length ($\mu\text{m} \pm \text{SD}$)	Known or putative cues
<i>a</i>	=	37.7 ± 16.1	1. Intrinsic polarity of the Ti1 cell bodies 2. Proximal increase in epithelial affinity
<i>b</i>	=	54.5 ± 12.1	Fe1 guidepost neuron
<i>c</i>	=	27.6 ± 10.1	1. Proximodorsal orienting factor 2. <i>m1</i> mesodermal cell (probable muscle pioneer ^b) 3. Other dorsally located cells, e.g., <i>m2</i> 4. Femur-trochanter segment boundary
<i>d</i>	=	55.7 ± 6.1	Tr1 guidepost neuron
<i>e</i>	=	38.2 ± 19.2	Distal segment boundary of coxa
<i>f</i>	=	73.7 ± 21.7	Cx1 guidepost neurons
<i>g</i>	=	127.6 ± 23.8	1. Unidentified, strong, proximal orientation factor 2. <i>p</i> cells

Total = 416.4^c*a*, *b*: *n* = 10; *c*-*g*: *n* = 11^a Segments are depicted in Figure 10.^b See text; cell depicted in Figure 5A (Ball et al., 1985).^c Total of mean segment lengths traversed by Ti1 growth cones.

Measurement of axon segments

The length of the *a* segment (Fig. 10) is measured as the distance between the point where the Ti1 axons emerge from their cell bodies to the point where they reorient toward the Fe1 cell. The length of the *b* segment is the distance between the reorientation toward the Fe1 cell to the point of contact with that cell, plus the length of axon running along the Fe1 cell to the point where the axon leaves it. The remaining axon segments are measured similarly.

Definition of cells, axes, and stages of development

The names of the various cells and limb axes are defined in the Materials and Methods section of Caudy and Bentley (1986), as are the stages of neuronal differentiation. General staging of embryogenesis is described in Bentley et al. (1979).

Results

Characteristic Ti1 Axon and Growth Cone Morphologies

Ti1 axons closely follow the initial growth cone routes

Ti1 axons are not displaced laterally, or "dragged" (Gunderson and Park, 1984), over the epithelial substrate by the motile growth cone. Distinct axon reorientations are regularly observed (Figs. 1, *B*, *D*, 2, *A*, *B*, 3C), indicating that the axons are firmly attached to the substrate, at least at the point of reorientation. In some cases, axon reorientations are observed immediately behind the growth cone (Fig. 3C), suggesting that the axons are attached to the substrate either as they are laid down, or very soon thereafter. In addition, Ti1 axons generally have distinct edges flattened against the epithelial substrate at all points along their length, an indication that they are attached all along their length.

Distinct reorientations occur at characteristic locations (see

Table 2. Percentage of Ti1 guidance by guidepost neurons between each pair of neurons on the path

Neuron pair	Ti1 guidance (%)
From Ti1 to Fe1	59
From Fe1 to Tr1	67
From Tr1 to Cx1s	66

below), and are observed at the same location both soon after the growth cone reorients and at substantially later stages. In Figure 1, *B* and *D*, a reorientation that is frequently observed about 30–50 μm proximal to the Ti1 cell bodies is present both when the growth cone is at the Fe1 cell and about 6 hr later when the growth cone is at the Tr1 cell. These observations indicate that the distinct reorientations observed in axon morphologies accurately reflect the location and direction of reorientations made by growth cones at earlier stages. Therefore, the morphologies of recently established, or "nascent," axons can be used to determine the precise routes taken by growth cones in a particular leg.

Ti1 axons follow a stereotyped route consisting of distinctly oriented segments

Observation of growth cone and nascent axon morphologies at various points along the path has shown that the overall Ti1 axon morphology consists of distinct, highly stereotyped segments of axon. In normal pathways, a series of straight, characteristically oriented segments of axon is observed (Figs. 1, *B*, *D*, 2, 3, and 7). In the most complex case of such normal pathways, the overall path is comprised of 7 distinct axon segments, which are schematized and labeled in Figure 10. The average lengths of these axon segments are given in Table 1 (see also Table 2). We will describe the most complex case first; variants in which certain segments are missing will be discussed later.

Segment *a*: The segment of initial outgrowth of the pair of Ti1 axons from their cell bodies is oriented proximally along the geometric axis of the tibia (Fig. 1, *A*, *B*, *D*; schematized in Fig. 10).

Segment *b*: The next segment is initiated by a single, distinct reorientation and is oriented in the proximoventral direction (Fig. 1, *B*, *D*). This segment extends straight in this direction to the Fe1 identified neuron and then extends along the dorsal edge of that neuron (Fig. 1*D*). The segment is terminated where the axons leave the proximal edge of the Fe1 cell and distinctly reorient (Fig. 1, *C*, *D*).

Segment *c*: On leaving the Fe1 neuron, Ti1 axons (Figs. 1*D*, 2*A*) and growth cones (Figs. 1, *B*, *C*, 5*A*, 6*A*) again distinctly reorient, this time in the proximodorsal direction. This reorientation is more acute than is necessary to orient proximally along the geometric axis of the limb (*cf.* drawing in Fig. 10), so that the *c* segment always is oriented dorsal to the proximally located Tr1 neuron (Fig. 1, *C*, *D*).

Segment *d*: This segment begins with a distinct reorientation from the *c* segment and is oriented proximoventrally (Figs. 1*D*; 2, *A*, *B*). The *d* segment extends directly to the Tr1 neuron and then along that neuron to its ventral edge (Figs. 4*D*, 5*D*).

Segment *e*: This segment is oriented circumferentially from its origin at the Tr1 cell (Figs. 2*B*, 3, 5*D*; see also Bentley and Caudy, 1983b). It extends straight ventrally and, as a result, is oriented toward a point that lies about 20 μm straight distal to the pair of Cx1 identified neurons (Fig. 3C). The *e* segment terminates with a distinct reorientation toward the Cx1 guidepost neurons.

Segment *f*: This segment is oriented toward the Cx1 neurons

Figure 1. T1l morphologies along the *a*, *b*, *c*, and *d* segments of the limb. *A*, The pair of T1l neurons has begun proximal axonogenesis, and their axons are extended straight proximally along the geometric axis of the limb. This forms the *a* segment (see text). The T1l growth cones typically have no branches on the *a* segment. The T1l cell bodies also are aligned along the proximodistal axis of the leg. Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *B*, The T1l cells are aligned along the limb axis and have extended axons proximally along the *a* segment. The axons distinctly reorient at a point (upward arrow) about 40 μ m proximal to the T1l cell bodies and then extend directly to the site (arrowhead) where the Fe1 cell arises. This forms the *b* segment (see text). From the Fe1 site, 2 clusters of filopodia (downward arrow) are extended proximodorsally in the direction of a pair of anti-HRP labeled cells (asterisk) in the epithelium. Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *C*, The T1l axons have extended proximally from their cell bodies and have contacted the Fe1 cell (solid arrowhead). Growth cones have numerous lateral filopodia, about 25–35 μ m in length, and have extended proximodorsally from the Fe1 cell, along the *c* segment (see text). A cluster of leading filopodia (downward arrow) is extended directly toward the large, lamellar *m1* cell (caret), which is labeled with anti-HRP. From the same growth cone, a branch is extended proximodorsally along a single filopodium (upward arrow) that is in direct contact with a labeled Tr1 cell (open arrowhead). Calibration bar, 50 μ m. *D*, The T1l axons have extended proximodorsally from the Fe1 cell (solid arrowhead) to a point (upward arrow at left) from which they have reoriented proximodorsally and extended directly toward the Tr1 cell (open arrowhead). This forms the *d* segment (see text). The T1l growth cones are spreading over the Tr1 neuron, which has begun axonogenesis in the ventral direction, along the distal segment boundary of the coxa. A cell (asterisk) labeled with anti-HRP lies near the point of reorientation, but is not precisely at it. A lamellum (star) is extended lateral to the T1l axons where they have crossed the tibia–femur segment boundary (see Caudy and Bentley, 1986). The T1l axons also make a distinct reorientation (arrow at right) toward the Fe1 cell after initially growing proximally along the *a* segment. Thus, in this leg the characteristic *a*, *b*, *c*, and *d* segments all are exhibited in the axon morphology of a growth cone that has extended from the T1l cell bodies to the Tr1 neurons. Calibration bar, 50 μ m.

(Fig. 3C), extends directly to and along them, and ends at their proximal edge.

Segment g: This segment begins at the proximal edge of the Cx1 neurons, extends straight proximally to the CNS without further reorientations (Figs. 4A, 7B), and ends with contact with the CNS.

In summary, in normal embryos the T1l axons are oriented in a characteristic direction at each point along their route to the CNS. Furthermore, since axon orientations reflect the orientation that the growth cone had as it grew, the growth cones apparently were specifically oriented at all points along the path, and thus apparently did not grow randomly at any point. Fur-

Figure 2. Ti1 morphologies along the *c*, *d*, and *e* segments. *A*, The Ti1 axons have extended straight from their cell bodies to contact a faintly labeled Fe1 cell (solid arrowhead). The Ti1 axons have their characteristic proximodorsal orientation along the *c* segment, to a point (arrow) where they distinctly reorient and extend directly to the Tr1 cell site, to form the *d* segment. A single, small branch also extends dorsally from the reorientation point (arrow). Calibration bar, 50 μ m. *B*, The Ti1 axons have extended straight from their cell bodies to a point from which lateral processes have contacted a faintly labeled Fe1 cell (solid arrowhead). From that point they extend along the *c* segment in the direction of the dorsally located *m1* cell (carat), which has labeled with anti-HRP in this leg (cf. Figs. 1C and 6C). The proximodorsal orientation is maintained to the point (arrow) where the Ti1 axons distinctly reorient and extend directly along the *d* segment to a faintly labeled Tr1 cell (open arrowhead). The Tr1 cell lies on the distal segment boundary of the coxa. After contacting that cell, the Ti1 growth cones have re-oriented circumferentially along the segment boundary (solid triangle), to begin forming the *e* segment (see text). As in *A*, Ti1 processes (several filopodia) extend straight dorsally from the point of reorientation (arrow). In this leg, a second dorsally located cell, *m2* (open triangle) has labeled with anti-HRP. A broad lamellar process is extended ventrally from the second dorsal cell. Calibration bar, 50 μ m.

Figure 3. Growth cone morphologies along the *e* and *f* segments. *A*, The Ti1 axons extend proximally to the distal segment boundary of the coxa (solid triangles), where their growth cones abruptly reorient and extend ventrally along the segment boundary. Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *B*, The same leg at higher magnification. Ti1 growth cones extend along the segment boundary to form segment *e*.

Figure 4. Ti1 morphologies and anti-HRP labeled cells along the *g* segment. *A*, The Ti1 growth cones (arrow) have nearly reached the boundary of the CNS (white square). The Ti1 axons are straight and unbranched along the *g* segment (i.e., the 60–70 μ m region straight distal to the growth cones), which lies between the Cx1 cells (unlabeled in this leg, but see Figs. 7 and 9) and the CNS. *Caret*, Efferent processes; *solid arrowhead*, Fe1 cell. Calibration bar, 100 μ m. *B*, The pair of Cx1 guidepost cells (*curved arrow*) has begun axonogenesis before the Ti1 growth cones have reached them. In this leg, another proximal (*p*) cell (*straight arrow*) on the final path has labeled with anti-HRP. This *p* cell lies about 15 μ m straight proximal to the Cx1 cells. The Ti1 growth cones have branches extended along the *c* segment. A single, very thin branch (*upward, narrow arrow*) is in contact with a labeled Tr1 neuron (not marked). *White square*, Boundary of CNS; *caret*, efferent fibers; *solid arrowhead*, Fe1. Calibration bar, 100 μ m. *C*, The Ti1 axons have reached the CNS boundary (white square). Two adjacent *p* cells (*arrows*) have labeled with anti-HRP. The more proximal of these cells is within one cell diameter of the CNS. The more distal of these *p* cells lies about 20–30 μ m proximal to the site where the Cx1 cells are located. *Caret*, Efferent fibers. Calibration bar, 100 μ m. *D*, The Ti1 axons have extended proximally to contact the Fe1 cell (*solid arrowhead*) and the Tr1 cell (*open arrowhead*), and have then turned circumferentially along the distal coxal segment boundary. The axons have turned proximally from the segment boundary and extended to the Cx1 cells (*curved arrow*). Two additional *p* cells (*straight arrows*) lie straight proximal to the Cx1 cells, so that together these cells form a continuous chain along the final path that reaches nearly to the CNS (white square). *Caret*, Efferent fibers. Calibration bar, 100 μ m.

thermore, since single, distinct reorientations typically initiate the characteristic segments, it would appear that at these points the growth cones made a discrete switch in the guidance cue that they were following. We will therefore consider these characteristic segments of the path with regard to known or possible guidance cues.

Growth cone interactions with guidepost neurons

The best characterized guidance cues for the Ti1 growth cones are identified guidepost neurons. In normal embryos the Ti1

(horizontal arrow). Three filopodia (*downward arrow*) are in direct contact with the Cx1 neurons (*curved arrow*). Calibration bar, 50 μ m. *C*, The Ti1 axons extend proximally to the Fe1 neuron (*solid arrowhead*) and then to the Tr1 neuron (*open arrowhead*). At the Tr1 neuron, they reorient circumferentially along the distal segment boundary of the coxa (*solid triangles*) to form segment *e*. They then make a single, distinct reorientation toward the Cx1 neurons (*curved arrow*) and extend directly to them to form the characteristic *f* segment (*downward arrow*). The Ti1 growth cones are at the Cx1 neurons and are wrapping branches and filopodia around one of them. Calibration bar, 50 μ m.

axons make direct contact with these cells and selectively dye-couple with them (Bentley and Keshishian, 1982a, b; Keshishian and Bentley, 1983a; Taghert et al., 1982). Deletion of the Cx1 guidepost cells has shown them to be a necessary cue for guidance along the normal path (Bentley and Caudy, 1983a, b).

Axon reorientations toward guidepost neurons

Three of the characteristic Ti1 axon segments—*b*, *d*, and *f*—are each oriented toward, make direct contact with, and then extend along the Fe1, Tr1, and Cx1 guidepost neurons, respectively. The point at which the Ti1 axons leave a guidepost neuron, after extending along it, typically is the precise point where the axons make a distinct reorientation onto the next characteristic axon segment (Figs. 1, *C*, *D*, 5*D*). Thus, that point of reorientation clearly defines the termination of the previous axon segment.

Growth cone branches and filopodia selectively wrap guidepost neurons

Growth cones do not simply extend along guidepost neurons that they have contacted. Frequently growth cones also profusely wrap labeled guidepost neurons with filopodia (Fig. 1, *C*, *D*, 3*C*)

Figure 5. Selective wrapping of unlabeled Fe1 and Tr1 guidepost cells by Ti1 branches and filopodia. *A*, The Ti1 axons have extended proximally from their cell bodies and have extensively wrapped an unlabeled Fe1 cell (solid arrowhead) with branches and filopodia. Two branches have extended beyond the Fe1 cell: one (upward arrow) directly toward the site where the Tr1 cell arises, and one (downward arrow) proximodorsally along the characteristic *c* segment toward the region where the *m1* cell occasionally labels with anti-HRP (see Figs. 1C and 6, C, D). A prominent, triangular-shaped lamellum (star) is extended laterally from the Ti1 axons, where they have crossed the tibia-femur segment boundary (see Caudy and Bentley, 1986). Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *B*, The Ti1 axons have extended proximally and have wrapped an unlabeled Fe1 cell (solid arrowhead) with branches and filopodia. At least one Ti1 axon (arrow) has extended further proximally and is wrapping an unlabeled Fe1 cell (solid arrowhead) with branches and filopodia, which extend around the complete circumference of the cell on the inner epithelial surface. Two Ti1 processes have extended beyond the Fe1 cell: a prominent branch (upward arrow) is extended directly to the site where the Tr1 cell arises, and several filopodia (downward arrow) are extended proximodorsally toward the region where the *m1* cells are typically located (see Figs. 1C, 6, B, C, D). Calibration bar, 50 μ m. *C*, The Ti1 axons have wrapped an unlabeled Fe1 cell (solid arrowhead) with branches and filopodia, which extend around the complete circumference of the cell on the inner epithelial surface. Two Ti1 processes have extended beyond the Fe1 cell: a prominent branch (upward arrow) is extended directly to the site where the Tr1 cell arises, and several filopodia (downward arrow) are extended proximodorsally toward the region where the *m1* cells are typically located (see Figs. 1C, 6, B, C, D). Calibration bar, 25 μ m. *D*, The Ti1 axons (arrow) have extended proximally and have contacted an unlabeled Tr1 cell (open arrowhead). Branches and filopodia have wrapped in both directions around the cell on the inner epithelial surface (the plane of focus), and additional filopodia are curving over its surface. The Tr1 cell lies on the distal boundary of the coxal segment (solid triangle) and a branch is extended ventrally along the segment boundary. Calibration bar, 25 μ m.

and occasionally spread lamellae over them (Fig. 1D). Branches also are observed to wrap over labeled guidepost neurons and to extend around the circumference of the cell where it contacts the epithelial substrate (Figs. 3, 4B).

The characteristic wrapping of filopodia and branches around neurons frequently reveals the presence and location of unlabeled guidepost neurons (Fig. 5). Filopodia selectively wrap single unlabeled cells at the Fe1 site (Figs. 5, A–C, 6A), the Tr1 cell site (Fig. 5, B, D) and at the site of the Cx1 cells (not shown). Branches extended around the circumference of unlabeled cells also are observed at the Fe1 site (Figs. 2A, 5, A–C, 6C), the Tr1

cell site (Fig. 5, B, D), and the site of the Cx1 cells (not shown).

The selective wrapping of Ti1 growth cone filopodia and branches around guidepost neurons suggests that growth cones specifically respond to contact with these cells. Furthermore, these characteristics allow Ti1 morphologies to be used as indicators of the presence of unlabeled guidepost cells on the path.

Ti1 morphologies and putative cues for the other characteristic segments

In any given embryo, Ti1 growth cones extending along the characteristic segments that are not oriented toward guidepost

Figure 6. Ti1 growth cone orientation along the *c* segment. *A*, The Ti1 axons have contacted an unlabeled Fe1 cell (solid arrowhead) and wrapped it with filopodia. At the Fe1 cell site, the pair of Ti1 growth cones has separated (long arrows), and each has independently oriented proximodorsally along a cluster of leading filopodia. The Tr1 cell (open arrowhead) has begun to label with anti-HRP and lies straight proximal to the Fe1 cell, but the Ti1 growth cones are oriented toward a region dorsal to the Tr1 cell, along the characteristic *c* segment. Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *B*, The Ti1 axons have extended proximally to the Tr1 cell site (open arrowhead) and are wrapping branches around an unlabeled cell at that site. A prominent branch (downward arrow) also has extended proximodorsally from the Fe1 cell site and has divided into 3 smaller branches that converge at the site of the *m1* cell (caret; cf. Fig. 1*C*). Curved arrow, Cx1 cells. Calibration bar, 50 μ m. *C*, The Ti1 axons have extended proximally from their cell bodies. Several filopodia (arrows) are extended parallel to one another from different sites on the Ti1 growth cone. They are extended proximodorsally toward the *m1* cell (caret), which is apposed to the inner epithelial surface. Anti-HRP-labeled particles extend radially from the *m1* cell and may reflect lamellar processes (cf. Fig. 1*C*), which the Ti1 filopodia may contact. Calibration bar, 50 μ m. *D*, In another leg, the *m1* cell (caret) has again labeled with anti-HRP, and several filopodia (arrows) extended proximodorsally from the Ti1 axons have made direct contact with it. At least 2 of the filopodia divide in 2 very near the point where they contact the *m1* cell. Calibration bar, 25 μ m.

neurons are as highly oriented as growth cones growing toward guidepost cells. Growth along each of the “nonguidepost” segments is initiated by a single distinct orientation (segment *a*) or reorientation, as though each resulted from a separate guidance cue. However, growth cones on these segments do not appear to be oriented by a specific, single cell at the termination point.

Segment a: proximal outgrowth from the Ti1 cell bodies

Ti1 growth cones and axons do not exhibit filopodial wrapping with labeled or unlabeled cells at the termination point of the *a* segment (Fig. 1, *A*, *B*). Also, the *a* segment varies in length by a greater amount than do the *b* and *d* segments (Table 1). These observations suggest that the *a* segment is not oriented

by a specific cell or feature at a fixed distance from the Ti1 cell bodies.

The *a* segment generally is oriented along the geometrical axis of the tibia, in which the Ti1 neurons arise (schematized in Fig. 10). The pair of Ti1 cell bodies are usually aligned along this axis (Figs. 1, 5, *A*, *B*, and 6*A*), so that their axons emerge from the proximal poles of the cell bodies and extend straight proximally until they reorient toward the Fe1 neuron (Fig. 1, *A*, *B*, *D*). Therefore, proximal axonogenesis along the *a* segment may be due to internal polarity of the Ti1 cell bodies. Alternatively (or in addition), there also is evidence that proximal guidance of the Ti1 growth cones in this region may result from proximal increases in the affinity of the substrate epithelium for growth cones (Caudy and Bentley, 1986).

Figure 7. Cx1 growth cone and axon morphologies in the *g* segment region. *A*, In some legs, the Cx1 neurons (curved arrow) pioneer the same pathway to the CNS (white square) that forms the *g* segment of the Ti1 axons. When the Cx1 growth cones extend proximally along this path, their growth cones are highly oriented straight proximally, even to the degree that filopodia tend to lie in very tight clusters (arrow). Calibration bar, 50 μ m. *B*, At a later stage, when the Cx1 (curved arrow) growth cones have reached the CNS (white square), their axons (arrow) are straight. The Cx1 axons have no lateral branches and very few lateral filopodia. Caret, Efferent fibers. Calibration bar, 50 μ m.

Segment c: proximodorsal reorientation from the Fe1 neuron

The *c* segment typically is initiated by a single distinct reorientation, suggesting a discrete switch in guidance cue. However, growth cones and axons on the *c* segment do not exhibit any specific interactions with labeled or unlabeled cells at the termination point of the segment. Furthermore, separated Ti1 growth cones (Fig. 6*A*) and filopodia (Fig. 6*C*) can orient proximodorsally from different points and extend roughly parallel. They are not oriented toward a single point in the region where subsequent reorientations typically occur. In addition, branches regularly extend proximodorsally beyond the point of reorientation toward Tr1 (Fig. 8). Also, branches occasionally (although very rarely) extend beyond the typical reorientation point and continue proximodorsally to the dorsal edge of the leg, where they appear to converge on an unlabeled cell (Fig. 6*B*).

In some legs, a specific cell, *m1*, transiently labels with anti-HRP in this same region at the dorsal edge of the leg (Figs. 1*C*, 6, *C*, *D*). Anti-HRP antibodies typically are specific for a cell-surface molecule on insect neurons (Jan and Jan, 1982), although

some other cell types also label (Jan and Jan, 1982; Shankland and Bentley, 1983). However, the *m1* cell does not appear to be a neuron. Unlike known neurons, it only occasionally labels with anti-HRP. Furthermore, even when it does label, it apparently does so only transiently, as labeled *m1* cells are not observed at later stages. Its bipolar morphology and axial alignment (Figs. 2*B*, 6*D*), its broad lamellae apposed to the inner epithelial surface (Fig. 1*C*), and its characteristic dorsal location suggest that it is one of a set of identified muscle pioneer cells in this region (Ball et al., 1985; Ho et al., 1983; Robert Ho, personal communication).

Filopodia regularly appear to be oriented specifically toward the *m1* cell (Figs. 1*C*, 6, *C*, *D*). On occasion, multiple filopodia directly contact this cell (Fig. 6*D*). Furthermore, after the Ti1 growth cones have reoriented and extended to the Tr1 cell, clusters of filopodia are regularly observed extending from the reorientation site toward the *m1* cell (Figs. 5, *A*, *C*, 8).

Other factors may also contribute to guidance along the *c* segment. The *c* segment does not always orient directly toward the *m1* cell (Fig. 8). Another cell, *m2*, occasionally labels in the same dorsal region as *m1*, and may contribute to growth cone guidance along segment *c*. The segment boundary between the femur and trochanter also develops near the termination point of the *c* segment (Caudy and Bentley, 1986) and may also contribute to Ti1 morphologies there. Furthermore, the distinct proximodorsal reorientation that initiates growth along the *c* segment occurs before the growth cones appear to be in filopodial contact with the *m1* cell or other dorsally located cells (Fig. 1*B*). Thus, there may be other, unknown cues involved in the initiation of growth along the *c* segment. However, the *m1* cell frequently does appear to influence the Ti1 growth cone in the terminal region of the *c* segment (Figs. 6*D*, 8). We conclude that *m1* contributes to branch extension and growth cone morphology on segment *c*, but that other factors may be involved.

Segment e: ventral reorientation from the Tr1 neuron

On leaving the Tr1 guidepost neuron, the Ti1 growth cones (Figs. 2*B*, 5*D*) and axons (Fig. 3; see also Bentley and Caudy, 1983*b*; and Caudy and Bentley, 1986) distinctly reorient circumferentially and extend ventrally to form the characteristic *e* segment. The guidance cue for this segment of growth has been identified as a limb segment boundary (Bentley and Caudy, 1983*b*), in particular, the distal segment boundary of the coxa (Caudy and Bentley, 1986). This segment boundary differentiates within the epithelial substrate. Once on this continuous circumferential cue, growth cones apparently extend circumferentially along it until filopodial contact with the proximally located Cx1 neurons distinctly reorients them proximally (Fig. 3; Bentley and Caudy, 1983*b*).

Segment g: proximal reorientation from the Cx1 neurons

On leaving the Cx1 guidepost neurons, the Ti1 axons and growth cones orient proximally, along the *g* segment. The *g* segment characteristically is very straight, without lateral branches of either Ti1 growth cones or axons (Fig. 4*A*).

Unlabeled axons from the Cx1 cells could provide a continuous cue for Ti1 growth cones on the *g* segment, since they sometimes pioneer the path to the CNS (Fig. 7). However, when the Cx1 cells pioneer the pathway, they too are very highly oriented and do not branch (Fig. 7). The lack of lateral processes extends even to the lack of lateral filopodia (Fig. 7). One possible cue for guiding the Ti1 and Cx1 growth cones along the *g* segment is a set of proximal cells, the "*p*" cells, which appear to form a nearly continuous chain there (Fig. 4, *B*, *C*, *D*). These cells sporadically label with anti-HRP and may be present in all legs. Growth cones occasionally exhibit a low degree of fil-

opodial wrapping of at least the most distal of these cells (Fig. 4B). However, growth cones (Fig. 7A) and axons (Figs. 4A, 7B) on the *g* segment typically exhibit no branch or filopodial wrapping of cells there. We conclude that there is a strong extrinsic factor that orients growth cones on the *g* segment and that the *p* cells may sometimes contribute to guidance there.

Normal variations in the axon path

Seven characteristic axon segments in the overall Ti1 path have been described. Different axon segments exhibit different degrees of variability in form and expression. The *g* segment always is present, and always extends straight proximally from the Cx1 neurons. The *f* segment also is always present, and always extends to the Cx1 neurons. However, the point at which the *f* segment begins can range from the point at the ventral edge of the Tr1 cells to any point more ventral along the distal segment boundary of the coxa, up to the point on the boundary that is straight distal to the Cx1 cells.

The *e* segment is totally absent in pathways in which the Ti1 axons extend directly from the Tr1 neurons to the Cx1 neurons. When it is present, it always is oriented straight ventrally along the segment boundary and ranges in extension from the Tr1 neurons to the point straight distal to the Cx1 neurons (Bentley and Caudy, 1983b; Caudy and Bentley, 1986).

The axon segments between the Ti1 and Fe1 neurons can vary both in the exact direction of orientation, and in whether they are present. The orientation of the *a* segment can range over about 30° of arc, between the line extending straight proximal along the geometric axis of the limb, and the line extending straight to the Fe1 neuron (schematized in Fig. 10, upper right). Axons occasionally extend straight from the Ti1 neurons to the Fe1 guidepost neuron (Figs. 2A, 5B). In such cases it is not possible to identify this straight segment as an *a* segment or a *b* segment, nor to determine if or where a switch in guidance cue was made. In other cases, the *b* segment is definitely missing (Fig. 2B; see also Caudy and Bentley, 1986, fig. 2, A, B), and the Ti1 axons do not directly contact a labeled Fe1 cell but pass dorsal to it.

Similarly, the axon segments between the Fe1 and Tr1 neurons can vary, both in whether they are present and in their exact orientation. Axons occasionally extend straight between these neurons, so that there is no apparent *c* segment (Fig. 5, C, D). However, even where there is no *c* segment, filopodial extension often is biased dorsally (Figs. 5C, 6C). In rare cases, axons can orient ventrally before reaching the distal coxa segment boundary and thus miss Tr1 (Fig. 4C; see also Caudy and Bentley, 1986); in these cases, both the *c* and *d* segments are missing.

The range of variation of axon pathways in normal embryos is schematized at the upper right of Figure 10. Other extreme variations occur in specially selected clutches of embryos where the Ti1 growth cones extend proximally before the Fe1 and Tr1 guidepost neurons have differentiated (Caudy and Bentley, 1986). In such embryos, straight axon pathways from the Ti1 neurons to the distal boundary of the coxa are regularly observed, and pathways frequently do not contact the Fe1 and Tr1 neurons once they differentiate (Caudy and Bentley, 1986, fig. 8F). Also, in these embryos growth cones in this region exhibit profuse branching and are not highly oriented (Caudy and Bentley, 1986).

Orientations and reorientations of Ti1 growth cones

Growth cone morphologies confirm the above observations for axon segment orientations. Ti1 growth cones in normal embryos have very few, if any, branches (Fig. 1). As a result, the orientation of the growth cone as a whole often can be clearly defined by the orientation of the axon or major branch trailing immediately behind the leading edge of the growth cone. Growth

Figure 8. Characteristic Ti1 branches and filopodial clusters at the site of reorientation toward the Tr1 neurons. *Upper diagram*, Schematic representation of a Ti1 neuron with axon segments along the *a* and *b* segments that has extended along the *c* segment to the point (carat) of reorientation toward the one Tr1 neuron. Branches are drawn in each of 3 directions characteristic of branch and filopodial cluster extension from that point: (1) directly toward the Tr1 neuron; (2) proximodorsally toward the *m1* cell; and (3) straight dorsally from the reorientation point (possibly toward the *m2* cell, or along the prospective femur–trochanter segment boundary). *Lower diagram*, The reorientation site (carat) lies at the center of a polar plot of the directions of Ti1 axons (solid triangles) and branches and filopodial clusters (open triangles) observed to extend from that point in 15 representative examples. Axons extended only to the Tr1 cell, never in the other directions. Branches and filopodial clusters characteristically are oriented in the direction of the *m1* cell, and also straight dorsally. No branches or clusters are observed in other directions, nor do cells label in other directions. The inwardly oriented solid triangles, lower right, indicate the directions from which the Ti1 axons approach the reorientation site along the *c* segment. Note that these directions are frequently not oriented directly toward the *m1* cell, although they may be in individual cases (e.g., Figs. 1C, 4B).

Figure 9. Average distance at which Ti1 axons reorient to the Fe1 and Tr1 neurons. The drawing schematizes a growth cone that has extended along the characteristic *a*, *b*, and *c* segments and is at the reorientation site that initiates the *d* segment that extends to the Tr1 neuron. The *r1* and *r2* distances are the average distances measured from the reorientation sites that initiate the *b* and *d* segments, respectively, to the point of contact with the Fe1 and Tr1 neurons.

cones at different locations along the path generally have the same orientation as do nascent axon segments observed at the same location in legs fixed at later stages. Thus, highly stereotyped orientations characterize both axons and growth cones at all points along the Ti1 pathway.

Disposition of growth cone branches

Growth cone branches do not appear to be extended randomly in normal embryos. They characteristically extend around adjacent guidepost neurons or are oriented along the segments of the ultimate Ti1 path (e.g., Figs. 1C, 4B, 5A, 6, A, B). Usually only single branches are oriented toward guidepost neurons (Figs. 1C, 5A); multiple branches are sometimes observed oriented to other cues (Figs. 4B, 6B). Branches that are extended lateral to the axon path are regularly observed at only 3 locations: (1) circumferentially at the distal segment boundary of the femur (Caudy and Bentley, 1986); (2) dorsally along the distal segment boundary of the coxa (Caudy and Bentley, 1986); and (3) at the site of reorientation that terminates the *c* segment (Figs. 2A, 8). Branch and filopodial extension at segment boundaries is described elsewhere (Caudy and Bentley, 1986). We conclude that in normal embryos, growth cone branches are extended only toward or along specific extrinsic guidance cues.

Disposition of growth cone filopodia

Filopodia of Ti1 growth cones fixed *in situ* are not disposed in a symmetrical fan at the leading edge of the growth cone, as is often seen in growth cone filopodia in culture (Bray, 1982; Letourneau, 1979). Rather, filopodia at the leading edge of Ti1 growth cones are predominantly (but not exclusively) oriented in the direction of extrinsic cues (Figs. 1, B, C, 2B, 3B, 5, A, C, 6, A, C, D, 7A, 8). Single filopodia are regularly extended toward distant guidepost neurons in regions where there are no adjacent filopodia of comparable length (Fig. 1C). In addition, clusters of filopodia (defined in Materials and Methods) are predominantly oriented to specific cues, along the *c* segment (Figs. 1, B, C, 5A, 6, A, C, 8), along the *e* segment (Fig. 2B), and along the *g* segment (Fig. 7A). We conclude that the prominent filopodia observed to contact guidepost neurons and the distinct filopodial clusters oriented toward other cues reflect growth cone response to specific extrinsic cues.

Growth cone reorientation process suggested by stereotyped Ti1 morphologies

The distinct reorientations of axon segments toward guidepost neurons suggest that growth cones also distinctly reorient toward

them. Although the dynamic process of growth cone reorientation cannot be observed in fixed tissue, the stereotyped morphologies of Ti1 growth cones suggest a particular sequence of growth cone response and reorientation to guidepost neurons.

Growth cones are observed that exhibit different degrees of orientation toward, and contact with, the Tr1 guidepost neurons: (1) growth cones oriented along the *c* segment, which have no processes oriented toward the Tr1 neuron, although that cell has begun to label with anti-HRP and is therefore definitely present (Fig. 6A); (2) growth cones oriented along the *c* segment, which have filopodia in direct contact with the Tr1 neuron but have no branches oriented toward that cell (not shown); (3) growth cones that have branches oriented directly toward the Tr1 neuron along a filopodium already in contact with it, although other growth cone branches and filopodial clusters also are present (Fig. 1C, 4B); (4) growth cones that have completely reoriented toward the Tr1 neuron, in that no branches are extended in other directions (Fig. 1D).

Similar observations have been made for growth cones that exhibit different degrees of orientation toward, and contact with, the Cx1 guidepost neurons: (1) growth cones that have oriented ventrally along the *e* segment and have no processes oriented toward the Cx1 neurons (Figs. 2B, 5D); (2) growth cones that have lateral filopodia in contact with the Cx1 neurons and also have branches extended further ventrally along the segment boundary cue for the *e* segment (Fig. 3, A, B); (3) growth cones that have a branch oriented toward the Cx1 neurons along filopodia in contact with them, although a branch is still further extended along the segment boundary (not shown here—see Bentley and Caudy, 1983b, fig. 4D); (4) growth cones that have completed reorientation toward the Cx1 neurons, with no branches oriented further along the segment boundary (Fig. 3C).

The above observations suggest that growth cone branches are guided directly to guidepost neurons by extending along filopodia that have already made contact with those neurons. Furthermore, reorientations toward guidepost neurons are not observed in situations where the growth cones could not be in contact with an unlabeled guidepost cell. Together these observations also suggest that growth cones reorient by withdrawing branches extended in other directions and continuing to extend along branches oriented toward, and in filopodial contact with, guidepost neurons. Thus, the point from which a branch is extended toward a guidepost neuron apparently becomes the point of distinct, single reorientation observed between 2 adjacent axon segments at a later stage.

Axon reorientations toward guidepost neurons occur at characteristic distances

To determine whether growth cones generally could contact guidepost neurons with filopodia from the points where they reorient, we have measured the distance at which axons reorient toward the Fe1 and Tr1 guidepost neurons. The averages of these distances are the *r1* and *r2* distances depicted in Figure 9. On the average, Ti1 growth cones reorient toward both the Fe1 and Tr1 neurons at a distance of about 30–35 μm . This distance often is spanned by Ti1 filopodia (Figs. 1C, 3B). Thus, these results support the hypothesis that the Ti1 growth cones contact distant guidepost neurons with filopodia, and then reorient directly toward them by extending branches along those filopodia.

Discussion

Ti1 morphologies reveal a sequence of distinct reorientations toward different cues

The characteristic orientations of nascent axon segments are the clearest indication of guidance by a sequence of different cues (Figs. 1, 2, 3, 4A, 7B; schematized in Fig. 10). However, the same orientations also are evident in the leading ends of growth

33-35%

Figure 10. Summary diagram of the features of the leg and Ti1 morphologies observed between the 33 and 35% stages. The pair of sibling Ti1 neurons have cell bodies that generally are aligned along the geometrical axis of the limb in the tibia (dashed line). The Ti1 growth cones emerge from the proximal poles of these cells and generally extend straight proximally along the limb axis, thus forming the characteristic *a* segment. After about 50 μ m of proximal growth, the Ti1 growth cones (in non-PPD clutches; cf. Caudy and Bentley, 1986) typically make a distinct reorientation in the proximoventral direction and extend directly toward and make contact with the Fe1 guidepost neuron at the midpoint of the femur (Fe), thus establishing the *b* segment. From the Fe1 cell, the Ti1 growth cones typically orient proximodistally toward the region where the *m1* cell labels occasionally, thus forming the *c* axon segment. After extending in this direction, the Ti1 growth cones again distinctly reorient in the proximoventral direction and extend directly toward and make contact with the Tr1 guidepost neuron. This establishes the *d* axon segment. After contacting the Tr1 neuron, the Ti1 growth cones and axons reorient circumferentially and extend along the distal segment boundary of the coxa. The *e* segment results from ventral growth along the segment boundary. The *f* axon segment then is initiated by a distinct reorientation toward and direct extension to the Cx1 guidepost neurons. After leaving the Cx1 cells, the Ti1 growth cones and axons extend proximally to the CNS, forming the *g* axon segment. A chain of cells, the *p* cells, sporadically and transiently labels with anti-HRP along the final path. The stippled region indicates the regions/orientations in which Ti1 branches normally occur. These include regions along the tibia-femur segment and trochanter-coxa segment boundaries (see Caudy and Bentley, 1986), as well as characteristic branches extended toward these *m1* and also straight dorsally from the reorientation site between the *c* and *d* axon segments. Lateral branches are not observed on the *g* segment. Ti1 growth cones and axons sometimes extend directly between neurons on the pathway, and the Ti1 axon morphology ranges between direct growth between these cells and the indirect, zigzag routes. This normal range of axon locations is indicated by the shaded regions adjacent to the axon segments depicted in the upper-right inset.

cones, as they extend along the path (Figs. 1, 2, 3C, 5A, 6, A, B, 7A, and 8). Furthermore, these same orientations are observed for individual branches of growth cones, which appear to extend exclusively to specific cues (Figs. 1C, 5A, 8). Finally, single filopodia (Figs. 1C, 4B) and filopodial clusters (Figs. 1, B, C, 2B, 7A, 8) also tend to orient in the direction of extrinsic cues.

Ti1 growth cones that grow through the same region of the leg before specific cellular cues have differentiated do not exhibit the same characteristic orientation of growth cone processes (Caudy and Bentley, 1986). Thus, these specific orientations of Ti1 processes are not due to intrinsic programs of neuronal morphogenesis but, rather, to response to extrinsic cues. (This also suggests that the distinct reorientations that have been analyzed here do not result from morphological deformation during tissue mounting.)

Guidance cues for the Ti1 pioneer growth cones

The known and putative cues for the 7 characteristic axon segments are listed in Table 1 (see also Table 2). Cell-ablation experiments have shown the Cx1 neurons to be necessary and sufficient cues for guidance along the *f* segment (Bentley and Caudy, 1983a, b). Ti1 growth cones and axons typically exhibit the same specific interactions with the Fe1 and Tr1 neurons as they do with the Cx1 guidepost neurons: (1) selective dye-cou-

pling (Bentley and Keshishian, 1982a, b; Taghert et al., 1982); (2) selective branch and filopodial wrapping; and (3) characteristic reorientations toward, direct extension to, and further extension along those neurons. Thus, Ti1 growth cones specifically interact with, and are guided by, each of the three guidepost neurons on the path.

Our results differ from those of Berlot and Goodman (1984), who concluded that the Fe1 neuron does not differentiate in time to influence the orientation or guidance of the Ti1 growth cones. In contrast, we observe characteristic wrapping of branches and filopodia around the Fe1 neuron and, more importantly, the distinct reorientation of the Ti1 growth cones and axons toward and along Fe1. We conclude that in many embryos, the Fe1 (and Tr1) neurons differentiate early enough to serve as guidepost neurons for the Ti1 growth cones and that in such embryos, the Ti1 growth cones both respond to, and are guided by, those neurons (see also Bentley and Keshishian, 1982a, b; Keshishian and Bentley, 1983a).

There are cases, however, in which proximal axonogenesis can begin before filopodial contact is established with the Fe1 neuron (Bentley and Caudy, 1983b; Berlot and Goodman, 1984). In such cases, proximal outgrowth is along the characteristic *a* segment and is oriented along the limb axis. Two possible guidance mechanisms for such proximal outgrowth have been suggested. The axial alignment of the Ti1 cell bodies suggests that there may be an internal polarity to the Ti1 neurons such that

growth cones emerge from their proximal poles and continue proximally (Bentley and Caudy, 1983b). Alternatively, there may be an extrinsic "limb axis cue," such as an adhesion gradient (Bentley and Caudy, 1983b; Berlot and Goodman, 1984). Evidence that the affinity of the epithelial substrate for growth cones increases proximally in this region has been presented elsewhere (Caudy and Bentley, 1986). Similar observations of directed outgrowth without guidepost cell contact have been made for pioneer axons in the wings of *Drosophila* (Blair and Palka, 1985; Blair et al., 1985; Schubiger and Palka, 1985).

Ti1 growth cones respond to cues of different cell types

The Ti1 growth cones respond to 3 different types of cells as they grow. The various guidepost cells are immature neurons. The guidance cue at the distal coxal segment boundary probably derives from (non-neuronal) epithelial cells, which are of ectodermal origin (Bentley and Caudy, 1983b; Caudy and Bentley, 1986). Finally, the *m1* cell to which the growth cones respond as they extend along the *c* segment is probably a muscle pioneer (Ball et al., 1985; Robert Ho, personal communication), and therefore of mesodermal origin.

Hierarchy of affinities

The Ti1 pioneer growth cones exhibit a hierarchy of affinity for their various guidance cues. The Fe1, Tr1, and Cx1 guidepost neurons are the dominant cues in that once filopodial contact is established with a guidepost neuron, growth toward that neuron is completed before the Ti1 growth cones reorient toward another cue.

Guidance along the *c* segment may be provided by the *m1* cell, and perhaps by additional cells in the dorsal region of the leg. Whether the *m1* cell is the cue for the initial reorientation along the *c* segment is uncertain. However, the Ti1 growth cones definitely respond to it (Fig. 6D): They regularly extend branches and filopodial clusters dorsally well beyond the point at which the growth cones reorient to the Tr1 guidepost neuron (Fig. 8) and, in very rare cases, can continue to the dorsal edge of the leg if they do not reorient to Tr1 (Fig. 6B). Despite these specific responses, the Ti1 growth cones normally reorient toward the Tr1 cell before they have completed growth to the *m1* cell and other cells in the dorsal region (Fig. 1, C, D). It therefore seems probable that the Tr1 cell is a higher affinity cue than the *m1* cell and other putative cues for the *c* segment.

A similar inference can be made of the relative affinities of the distal coxal segment boundary and the Cx1 guidepost neurons. The segment boundary can provide a high-affinity cue for pioneer growth cones. Growth cones on the segment boundary can follow a very straight circumferential path along it for as much as 180° of arc around the circumference of the leg (Bentley and Caudy, 1983b). However, Ti1 axons always are observed to reorient away from the segment boundary and to extend directly to the Cx1 neurons, although branches here also extend beyond the reorientation point, and growth on the segment boundary clearly could have continued (Caudy and Bentley, unpublished observations). Thus, the Cx1 neurons appear to be a higher affinity cue than the segment boundary.

The reorientation from the *a* segment toward the Fe1 neuron may or may not reflect a hierarchy of affinity. Orientation along the *a* segment may be due to an intrinsic mechanism rather than an extrinsic cue, in which case the reorientation toward Fe1 would not reflect a difference in affinities between extrinsic cues.

The reorientations that occur as growth cones leave a guidepost neuron do not reflect orientation along a cue of higher affinity, since no further guidance by that neuron is possible. The subsequent continuation of growth apparently reflects the absence of a specific "stop" signal for the Ti1 growth cones,

rather than reorientation along a higher affinity cue. Thus, for the Ti1 growth cones, growth along a sequence of cues of different affinities does not require a continuous increase in affinity of cues in order for navigation to continue.

Molecular aspects of the affinity hierarchy

The differences in affinity exhibited by the Ti1 growth cones for their various cues probably result from the differential expression of affinity molecules by them. There could be a difference in the degree of expression of a single type of affinity molecule on the different cues, or there could be different molecules for some or each of the cues. Also, these affinities could be adhesive in nature or could involve some active form of biochemical signaling (see also Caudy and Bentley, 1986). The differential expression of adhesion molecules on the cues might explain the observed preferential disposition of filopodia toward specific cues. If the cues are adhesive for filopodia, randomly extended filopodia would be preferentially retained at cues, while other filopodia are withdrawn (Bray, 1982; Letourneau, 1983). However, it is also possible that the various cues are adhesive for filopodia, but that some or all cues also interact actively. The selective establishment of dye-coupling with the guidepost neurons (Bentley and Keshishian, 1982a, b; Keshishian and Bentley, 1983a; Taghert et al., 1982), indicates that these cells, at least, may undergo more than simple adhesive interactions with pioneer growth cones.

Interestingly, cues of all 3 cell types label with anti-HRP, which generally is specific for surface molecules on neurons (Jan and Jan, 1982). Although the *m1* cell apparently is a mesodermal cell, it still labels with anti-HRP. Similarly, the epithelial cells at segment boundaries also tend to label with anti-HRP (Caudy and Bentley, unpublished observations), and segment boundaries form high-affinity cues for the Ti1 growth cones. Furthermore, the observed differences in affinity correlate with the degree of expression of the molecular binding site for anti-HRP. The highest affinity cues are the guidepost neurons, which all label strongly and consistently with anti-HRP by the 35% stage. The lower affinity cues such as the *m1* cell and segment boundaries label weakly and inconsistently. Thus, the anti-HRP binding site might be a molecular cue for neuronal recognition, or it might be a surface molecule whose expression is linked to the expression of such a cue.

Characteristics of growth cone steering

Growth cones steer by selective branch extension

The process of growth cone reorientation toward guidepost neurons was inferred from Ti1 morphologies consistently observed in fixed tissue (see Results). Growth cone branches are observed to extend along filopodia already in contact with guidepost neurons (Fig. 1C). The growth cone apparently reorients by extending along that branch and by withdrawing branches extended in other directions.

This process is supported by the additional observation that growth cone branches are not only directly extended to specific cues, but are also exclusively extended to cues (Fig. 8). Thus, the observed extension of a branch toward a guidepost neuron probably in no case is due to random growth. This strengthens the interpretation that branches are selectively extended along filopodia in contact with guidepost neurons in response to that contact.

An analogous sequence of growth cone reorientation toward cues other than guidepost cells also can be inferred from stereotyped Ti1 morphologies. For example, Ti1 growth cones that have reached the Fe1 cell typically extend clusters of filopodia proximodorsally, along the *c* segment (Fig. 1B). At slightly later stages, Ti1 growth cone branches selectively extended along the *c* segment are observed (Fig. 1C). At later stages, nascent axon

segments are observed that are oriented along the *c* segment (Fig. 1D).

Thus, both for reorientations toward guidepost neurons and for reorientations to other cues, a distinct reorientation in the nascent axon pathway is established by selectively extending a branch along, or to, the cue for the next segment. This selective extension of branches is in contrast to the extension of multiple, variously oriented branches in clutches of embryos in which the T1l growth cones extend proximally before the Fe1 and Tr1 neurons and other specific cues have differentiated (see discussion of "PPD clutches" in Caudy and Bentley, 1986).

Selective branch extension along oriented filopodia

Filopodia in fixed tissue are not randomly oriented in advance of growth cone branches, but themselves tend to be oriented to the same cues. The disposition of filopodia that precede branches varies widely, apparently depending on the nature of the cue being followed. Clusters of filopodia oriented directly ahead of branches are regularly observed on the *c* segment (Figs. 1C, 6A), on the *e* segment (Fig. 2B), and on the *g* segment (Fig. 7A). The width of the cluster varies, being very narrow on the *g* segment and relatively wide on the *c* and *e* segments.

Filopodial dispositions on the segment boundary cue for the *e* segment vary greatly, perhaps due to variation in the degree to which the boundary has differentiated at the time of growth along it. Filopodial clusters are regularly observed there (Fig. 2B), but branches sometimes extend along it without preceding filopodia (Fig. 5D). Growth cones also have been observed to orient along the segment boundary following a single thin process, either a single filopodium or a very thin branch (see Bentley and Caudy, 1983b, fig. 4D; see also Shankland, 1981, for a discussion of similar growth cone morphologies).

In some embryos, the T1l growth cones extend proximally before the Fe1 and Tr1 guidepost neurons have differentiated, apparently in response to a proximal increase in the affinity of the epithelial substrate within segments (see discussion of "PPD clutches" in Caudy and Bentley, 1986). This cue appears to be distributed over the epithelial substrate, and in PPD clutches the T1l filopodia appear to be more randomly oriented over the epithelium. In general, the presence and disposition of filopodia extended ahead of growth cone branches may depend on (1) whether growth is along a continuous cue (such as the segment boundary) or (2) is toward a distant cue (such as guidepost neurons), (3) the relative affinity of the cue, and (4) the degree to which the cue is localized.

Distinct, single reorientations imply distinct switches in guidance cue

The single, distinct reorientations observed between nascent axon segments suggest that a discrete switch was made between guidance cues. This interpretation is supported by the further observation that at earlier stages, growth cone branches not only are oriented specifically to cues, but are also oriented exclusively to cues. At no stage are branches extended in intermediate (non-cue) directions. Thus, at no stage or location does the direction of an individual branch appear to reflect the sum influence of multiple cues. This further suggests that a growth cone reorients by making an abrupt "switch" or commitment to extension along the branch oriented to the highest affinity cue, rather than by a gradual reorientation as the growth cone progresses.

Direct filopodial contact guides branch extension to guidepost neurons

The extension of growth cone branches toward distant guidepost neurons apparently results from direct filopodial contact. The consistent observation of growth cones with different degrees of orientation toward, and contact with, the Tr1 and Cx1 guidepost

neurons suggests a process by which T1l growth cones reorient to guidepost neurons (see Results). Direct filopodial contact apparently triggers selective branch extension toward guidepost neurons, as well as the eventual withdrawal of other branches previously extended to other cues.

Several further observations support this hypothesis. Branches are not observed that are oriented in the direction of guidepost neurons but are clearly not, or could not be, in direct filopodial contact with them. In addition, the distinct reorientations observed between nascent axon segments toward the Fe1 and Tr1 guidepost neurons both occur at a distance of 30–35 μm , which corresponds to the filopodial reach of the growth cone (Bentley and Caudy, 1983a, b; Bentley and Keshishian, 1982a, b; Keshishian and Bentley, 1983a; Taghert et al., 1982). The fact that reorientations toward both of these neurons occur at about the same distance suggests that the same guidance mechanism occurs in both cases. Furthermore, filopodial contact with the Cx1 neurons from growth cones on segment *e* also is frequently observed at this distance (Fig. 3B). Therefore, direct filopodial contact is a likely mechanism for selective branch extension, and commitment to growth, to guidepost neurons.

Single filopodia can direct growth cones to guidepost neurons

At all locations on the path, branches tend to be oriented to specific cues (e.g., Fig. 8). Therefore, branches oriented in the direction of a guidepost neuron are probably selectively extended to some cue in that direction rather than randomly extended. The observation of direct contact by a single filopodium (Fig. 1C) suggests both that the neuron is the cue, and also that single filopodial contact mediates selective branch extension. (It also is possible that the initiation of branch extension requires multiple filopodial contact, but, if so, the additional filopodia must be withdrawn as the branch extends along a single filopodium.)

T1l growth cones generally extend single branches toward guidepost neurons. These single branches typically are extended along single filopodia that are in direct contact with those neurons. Branches in fixed tissue are observed at several apparent stages of extension along single filopodia to guidepost neurons: (1) Filopodial contact is made before branch extension begins (Fig. 3B); (2) a branch is extended partway along a single filopodium that is in direct contact with a guidepost neuron (Fig. 1C); (3) a branch is extended completely to the site of a guidepost neuron (Fig. 5A); (4) the growth cone has reoriented and has reached the guidepost neuron (Fig. 1D).

The progressive development of a branch extended along a single filopodium also is suggested by the observation of extremely thin branches (Fig. 4B) extended to distant guidepost neurons, as well as much thicker branches at presumably later stages (Fig. 5A). Although branch extension to guidepost neurons may sometimes occur along multiple filopodia, single filopodial contact regularly, and perhaps typically, directs branch extension to them.

Single filopodia can reorient growth cones in the presence of more prominent processes

Growth cones reorient toward guidepost neurons along single filopodia, even though multiple filopodia, or even prominent branches, still are preferentially oriented to the previous cue. Although these more prominent processes are eventually withdrawn, and may be in the process of withdrawal at the time of reorientation, they are present throughout the reorientation process, and sometimes for substantial periods afterward. For example, the multiple filopodia and/or branches that are selectively oriented along the *c* segment are not only still present after the T1l growth cones have reached the Tr1 neuron, but

sometimes also after they have even reached the CNS (Fig. 4, A, D). Thus, contact with a guidepost neuron by a single filopodium consistently will reorient a growth cone that is still oriented to the previous cue.

Implications for models of growth cone steering

How does single filopodial contact with a guidepost neuron lead to selective branch extension and commitment to growth along that specific filopodium while many other filopodia remain preferentially oriented to the previous cue? Two basic types of growth cone steering models have been proposed. In their most extreme form, "passive adhesion" models propose that contraction forces generated within filopodia cause the retraction of nonadherent filopodia and produce traction forces along adherent filopodia that "pull the growth cone across the substrate" (Gundersen and Park, 1984). In such models, each filopodium that is selectively retained by adhesive contacts would contribute to the overall motive force exerted on the growth cone, and the final direction of movement would be in the direction of the vector sum of such forces. In contrast, "active" models involve biochemical response to contact with guidance cues, perhaps by regulating cytoskeletal conformation (Cooper and Schliwa, 1985), which might amplify relatively small differences between cues.

A problem with passive adhesion models is that differences in adhesion may be too weak to steer growth cones, let alone steer them along single filopodia. Growth cones in culture can extend directionally on growth factor-conditioned substrates where the spatial differences in the adhesivity of the substrate are relatively small (about 20%; Gundersen and Park, 1984), suggesting that other processes may be necessary to steer growth cones, or at least to amplify adhesion differences that are present (Gundersen and Park, 1984).

Aligned microfilaments have been observed at the bases of filopodia fixed in culture (Letourneau, 1979). Two processes have been suggested by which relatively small adhesion differences might lead to growth cones steering along such aligned microfilaments: (1) Traction forces along filopodia "may be directed to pull forward intracellular structures that interact with actin filaments" (Letourneau, 1979; see also Bray, 1979), rather than to pull the whole growth cone; or alternatively, (2) forces generated along actin filaments within filopodia "may be transmitted to microtubules and other structures [within the growth cone] to determine their positions in the growth cone margin" (Letourneau, 1983). The directed orientation of microtubules could then produce directed branch extension by an active process of adding monomers to the microtubule ends. In this way an active process would amplify an initial response that was based on relatively weak differences in adhesion.

However, the same alignment that mediates these two processes could also arise from an active response to a nonadhesive cue. For example, a filopodium in contact with a guidepost neuron might send a second messenger down its length to its base, where it could, for example, cause a localized contraction of the actomyosin known to be present there. Pioneer growth cones selectively dye-couple with guidepost neurons (Bentley and Keshishian, 1982a, b; Keshishian and Bentley, 1983a; Taghert et al., 1982), and filopodia and neuronal cues also undergo other active interactions (Bastiani and Goodman, 1984). Thus, active responses to filopodial contact with guidepost neurons are a reasonable possibility.

Does differential adhesion mediate the steering response of the T1l pioneer growth cones? Recall that multiple filopodia and/or branches are observed to be selectively oriented toward the *m1* cell while the growth cone is in the process of reorientation to Tr1 and are often observed well after the completion of that reorientation. According to strictly passive adhesion models of steering, these processes should have been selectively retained by adhesive contacts. As a result, they also should exert

traction forces on the growth cone that influence its direction of growth. However, no such influence is observed, nor do T1l growth cones steer in the direction of the greatest number of selectively retained filopodia.

Thus, the T1l growth cones do not appear to be steered by the vector sum of filopodial forces acting on the growth cone. The growth cone neither advances nor is it pulled as a unit along the epithelial substrate, since filopodial clusters and prominent branches remain at their original locations for significant periods of time after the growth cone reorients. Rather, the growth cone can respond independently to multiple cues, although it consistently reorients to the cue of highest affinity, and eventually withdraws branches previously extended to cues of lesser affinity.

Finally, growth cones also appear to make a distinct switch in the cue to which they are steering, and probably can do so on the basis of contact by a single filopodium. Our results are not consistent with a strictly passive adhesion model of growth cone steering, but they are consistent with models of steering by differential adhesivity of cues in which intracellular events amplify adhesive interactions sufficiently for growth cones to reorient along single filopodia. Alternatively, the results are also consistent with purely active models of steering that do not involve adhesion differences between cues, but, rather, are mediated solely by active guidance molecules.

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