The Oblique Muscle Organizer in Hirudo Medicinalis, an Identified Embryonic Cell Projecting Multiple Parallel Growth Cones in an Orderly Array

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The oblique muscle layer in the leech body wall is built upon the processes of a unique identified embryonic cell, the Comb- or C-cell. Each C-cell is composed of a spindle-shaped soma that projects approximately 70 parallel processes through the developing body wall at an angle oblique to the long axis. The morphogenesis of this cell and the navigation of its growth cones were examined by intracellular dye filling and antibody staining. At the earliest stages described each C-cell had about six processes, with those near the center of the cell oriented obliquely. As processes were added at the axial ends of the soma they often projected along previously developed longitudinal or circular muscle founder cells and then secondarily aligned themselves parallel to the older processes from the same C-cell. All growth cones initially extended to a particular location in the body wall, where they ceased growing until all 70 processes had been added (over the course of about 5 days). As adjacent segmental homologs met, their growth cones intermingled, eventually sorting out in a parallel array. When one of these cells was ablated early—but not later—in development, the remaining adjacent segmental homologs expanded into the vacant territory, consistent with a hypothesis of mutual avoidance between segmental homologs. Most processes that expanded into the experimentally induced vacancy remained correctly oriented and parallel; the few exceptions projected instead along the mirror-image trajectory. Thus, expression of specific avoidance between adjacent C-cell processes is developmentally regulated and functions as a guidance mechanism in vivo, in that it serves to restrict possible trajectories. After aligning its growth cones, each cell stopped adding processes and the processes rapidly extended in concert along relatively precise trajectories. Processes of contralateral homologs cross to form the orthogonal grid used as a scaffold by myocytes to form the oblique muscles. The advancing fronts of growth cones reached the dorsal midline at about the same time as body closure occurs (at about Embryonic Day 20) at which time the C-cells became granular, lost processes, and presumably died. This sequence of developmental events is consistent with temporal and spatial regulation of different morphogenetic strategies, including—but not limited to—specific avoidance, and further suggests testable hypotheses of mechanisms of growth cone navigation in the intact embryo.

INTRODUCTION

During embryogenesis organized tissues are built up largely by cell movements. The growing tips of neurons (growth cones) represent a specialized case of cell movement in which the soma remains in a fixed position while processes extend (Trinkhaus, 1985). In both vertebrates and invertebrates this navigation is often very precise and is thought to be underlie, to a large extent, the development of specific connections between neurons and their targets (Kater and Letourneau, 1985). By virtue of their roles in motility and specific pathfinding, growth cones have been an object of intense scrutiny. These structures provide much of the motile machinery for process elongation (Bray, 1987; Heidemann et al., 1990; Letourneau et al., 1987; Mason, 1985) and are thought to mediate navigation by interacting with environmental cues (Bastiani et al., 1985; Chiquet et al., 1988; Eisen, 1988; Fallon, 1985; Hammarback and Letourneau, 1986; Jay and Keshishian, 1990; Jessell, 1988; Kowada et al., 1990; Letourneau, 1982; Letourneau et al., 1990; Tosney, 1988).

It is clear that growth cones are capable of using a variety of guidance cues, including differential adhesivity (Gundersen, 1987; Harris, 1973; Letourneau, 1975; Goodman et al., 1984), chemotaxis (Berg, 1984; Greene et al., 1984; Levi-Montalcini, 1976), haptotaxis (Michler and Meyer, 1980; Silver and Sidman, 1980; Binger et al., 1979), and galvanotaxis (Borgens et al., 1980; Patel et al., 1985). Underlying these may be intrinsic constraints on polarity (Caceres and Kosik, 1990). It also seems that growth cones in vivo use multiple mechanisms (Rhaar and Paika, 1985; Caudy and Bentley, 1985a,b; Landmesser et al., 1988), perhaps simultaneously or in different combinations at different times and locations. Thus, examinations of growth cone-mediated morphogenesis are often complicated, and manipulations of in vivo systems can lead to ambiguous results.

Equally intriguing is the idea that growth cones may use specific inhibitory interactions to restrict the direc-
tionality of growth (Keynes and Cook, 1990; Patterson, 1988) and hence guide their navigation. Such a mechanism has been implicated for the interaction between retinal ganglion cell growth cones and tectal membranes in vitro (Bonhoeffer and Haf, 1980; Stahl et al., 1990; Vielmetter and Staermer, 1989; Walter et al., 1987) and between different neuronal types (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987; Moorman and Hume, 1990). The importance of nonpermissive guidance cues has also been suggested for at least a few in vivo situations (Carpenter and Bastiani, 1991; Tosney, 1991; Tosney and Oakley, 1990), and self-inhibition has long been postulated as a mechanism to explain the low degree of overlap in innervation territories from different branches of the same neuron as well as to explain why multiple processes from the same cell usually do not fasciculate or innervate overlapping areas (Kramer and Stent, 1985; Kramer et al., 1987; Wallace, 1984). While these studies point out the general importance of cell-cell and cell-matrix interactions (or interactions of neuronal precursors) have been shown to play important regulative functions in cell birth (Baptista and Macagno, 1988b; Baptista et al., 1990), cell death or transformation (Blair et al., 1990; Macagno and Stewart, 1987; Shankland, 1984; Shankland and Martin, 1989; Stuart et al., 1987), neuronal migration (Stuart et al., 1989; Torrence and Sturt, 1986; Torrence et al., 1989), and selective axon extension and retraction (Baptista and Macagno, 1988a; Glover and Mason, 1986; Gao and Macagno, 1987a,b; Gao and Macagno, 1988; Jellies and Kristan, 1988a; Loer and Kristan, 1988a,b; Loer et al., 1987; Wallace, 1984). While these studies point out the general importance of cell-cell and cell-matrix interactions in leech development, none of the leech studies have directly addressed the issue of whether these interactions are driven by events in the growth cones.

Here we describe the development of an identified embryonic cell—the Comb- or C-cell—in the medicinal leech, and we have used its development to investigate the interaction of growth cone guidance mechanisms. Each C-cell projects about 70 parallel processes along oblique trajectories, and each embryo contains segmental repeats of this cell for a total of at least 44 homologous cells (one pair in each of 21 midbody segments and a single pair in the prostomium). We have shown previously that myocytes gather around and elongate along the processes of C-cells to form the oblique muscle layer in the leech body wall (Jellies and Kristan, 1988b). Its development is therefore of interest for two reasons: First, it is a defined tissue organizer (in that it is an essential causal link in the developmental sequence that leads to histogenesis); and second, it seems to be an excellent model for investigating how growth cones navigate in vivo. In this paper we emphasize the navigation of C-cell growth cones because these dominate C-cell morphogenesis. We have been able to identify what appear to be important developmental features that implicate a number of qualitatively different morphogenetic mechanisms. We have also shown that interactions between growth cones of segmentally adjacent C-cells help to establish the orientation and spacing of processes at segmental boundaries. Thus it appears that these cells use multiple mechanisms for guiding the outgrowth and orientation of their processes during normal development.

MATERIALS AND METHODS

Animals

Embryos of the medicinal leech, *Hirudo medicinalis*, were obtained from a breeding colony, maintained as previously described (Jellies et al., 1987) at 23–25°C, and staged in days of development (Fernandez and Stent, 1982). Further divisions of staging encompass early, middle, and late corresponding to approximately 8-hr intervals.

Intracellular Staining

Embryos were dissected and viewed using DIC optics as previously described (Jellies and Kristan, 1988b). Cells were identified visually, impaled with microelectrodes made from thin-walled omega dot capillaries, and filled with either 3% aqueous Lucifer yellow (LY) or 4.5% horseradish peroxidase (HRP, Type VI) dissolved in 0.2 M KCl with 2% fast green (all reagents from Sigma). The large nucleus with its single nucleolus was invariably the best feature to guide penetrations since it is more prominent than any other nucleus in the body wall. The C-cell was identified and filled intracellularly with dye as early as Day 7 of embryogenesis and as late as Day 19–20. We examined the development of this morphologically complex cell by filling cells with dye in adjacent segments at 24-hr intervals, concentrating on segments 4–16.

Lucifer yellow was injected using constant hyperpolarizing current (0.2–1.0 nA) for 15–90 sec. Preparations were kept at room temperature (23°C) for 5–10 min after the last penetration to allow the dye to diffuse. They were then fixed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30–60 min or overnight. Following fixation the preparations were rinsed with PBS, dehydrated, cleared in methyl salicylate, and mounted in Fluoromount (Gurr) or kept in cold PBS for further processing.

HRP was pressure injected (Jellies et al., 1987) using
several 2- to 3-sec pulses at 2–10 psi. Following the last injection preparations were placed in L-15 medium on ice for 60–90 min to allow diffusion of the enzyme. They were then pinned flat, fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) on ice for 30–60 min, and then rinsed in PBS. Preparations were changed to 0.1 M Tris buffer (pH 7.4) through several rinses, permeabilized in 0.5% saponin in Tris for 15–30 min, rinsed in Tris, and then incubated with cold 0.5 mg/ml DAB in Tris for 15–20 min. Staining was intensified by adding CoCl₂ (3–5 µl 0.25 M CoCl₂ in Tris/ml DAB) during the last 5 min of incubation. Reaction product was produced using H₂O₂ (Muller and Carbonetto, 1979; Gillon and Wallace, 1984), and the preparations were rinsed in cold Tris, dehydrated in ethanol, cleared in methyl salicylate, and mounted in Permount.

Antibody Staining

Two antibodies were used in this study, a rabbit-serum antibody directed against LY (Taghert et al., 1982), provided by Drs. C. Goodman and J. Kuwada, and a monoclonal antibody (Lan 3-14) that recognizes leech muscle (Zipser and McKay, 1981), provided by Dr. B. Zipser. Tissues processed with either antibody were fixed overnight in cold 4% paraformaldehyde as above and then rinsed in PBS.

Anti-LY serum was diluted 1:1000 in PBS that contained 1% BSA and 1% Triton X-100. Preparations were incubated in this solution overnight at 4°C, rinsed for 1 hr in several changes of PBS, and then incubated with HRP-conjugated goat anti-rabbit IgG (Cappel) diluted 1:1000 in PBS that contained 1% BSA, 1% Triton, and 2% goat serum. The HRP was developed as previously described (Jellies et al., 1987); the preparations were then dehydrated, cleared, and mounted in Permount. Lan 3-14 was employed as previously described (Jellies and Kristan, 1988a,b) using a rhodamine-conjugated secondary antibody.

Sectioning

In a few cases, selected preparations were embedded in Epon following dehydration in a graded series of ethanol solutions. Preparations using HRP were cleared in propylene oxide before embedding. Serial 2- to 4-µm sections were cut on an LKB microtome and most sections were counterstained with toluidine blue before mounting in Permount.

Measurements and Documentation

Measurements were made with an ocular micrometer from fixed and cleared whole-mounts viewed through the translucent epidermis, from photographs, or from images superimposed onto a calibrated rule using a drawing tube (Leitz). Most measures were normalized to defined landmarks in the body wall (Results) to allow for variations in embryo size and dissection as well as to allow measures from embryos of different ages to be compared. The exception to this was the number of processes/C-cell. At early stages, an extension was counted as a process if it was longer than the longest nuclear diameter, at later stages (Embryonic Days 11–13) processes were counted as previously described (Jellies and Kristan, 1988b). The quantitative descriptive results in this study were derived from a sample population of 1122 individually dye-filled C-cells in 98 embryos from Embryonic Day 7 (E7) to E13. A roughly proportional number of cells were filled in preparations staged E14–E18, which are presented as qualitative descriptive summaries.

Preparations were photographed on a Wild/Leitz Laborlux S compound microscope using transmitted blue-filtered light and Kodak Technical Pan film, Kodak T-Max (ASA 400), or using appropriate filter sets for fluorescence on Kodak Ektachrome color slide film as previously described (Jellies and Kristan, 1988b).

RESULTS

The medicinal leech contains 32 segments. The first four and the last seven segments are fused to form a head and tail, and the remaining 21 are similar to one another: they are termed midbody segments and numbered accordingly (Ort et al., 1974). The C-cell is an identified cell in that there is only one bilateral pair per segment, located in a constant position relative to other morphological features in each midbody segment from a given individual and in the same location in different individuals (Jellies and Kristan, 1988b). The C-cell soma
is located in the ventral body wall, about midway between the nephridiopore and the margin of the ganglonic primordia, with the nucleus (which is approximately the midpoint of the C-cell) located at about the anterior margin of the ganglionic primordia (Fig. 1). Within the body wall this cell is embedded between two discrete layers of muscle, the more superficial circular muscles and the deeper longitudinal muscles (Fig. 2).

**C-Cells Develop in an Anterior–Posterior Gradient**

Segments are established in leeches by the successive laying down of segmental precursor cells; this procedure starts at the anterior end, which means that the more anterior segments are older than the posterior ones (Stent et al., 1982). Therefore, one can examine the development of C-cells either by filling the C-cells in one par-
C-Cells Elongate while Adding and Orienting Processes

Mature C-cells each possess about 70 parallel processes (Jellies and Kristan, 1988b), 35 on each side. At the earliest developmental stages at which these cells could be identified, the C-cells had many fewer processes, as few as three on each side (Figs. 3A and 5). Two observations support the idea that processes are added primarily at the anterior and the posterior ends of these cells. First, at progressively later stages the anterior and the posterior ends of the cells were further from the central nucleus, indicating that they elongated fairly symmetrically along the anterior-posterior axis (Fig. 3). Second, even at the early stages, only the longest processes at the central portion of the cells were obliquely oriented. Consistently, the processes located at the anterior and the posterior ends were shorter and much more variable in orientation.

Processes were progressively added to each C-cell through E11 (Fig. 4, solid bars), remaining stable thereafter. Most of the processes originated from the cell's soma, but a small number of them were apparently added as branches of existing processes. Such branch points were seen in older C-cells (examples can be seen in Figs. 1 and 10B, and Jellies and Kristan, 1988d), and what appeared to be incipient branch points were observed on some of the growth cones of young C-cells (Fig. 5).

A third possible mechanism for adding processes is suggested by the observation of membranous veils between parallel tracks of thickened cytoplasm (Fig. 5). These veils were more numerous and larger in the younger cells than those in the older cells, which suggests that a few processes might arise as a gradual "un-zipping" of previously extended and oriented processes. Alternatively, this may represent growth cone...
advance by veil extension similar to that thought to occur elsewhere (O'Connor et al., 1990).

The sequence of adding processes to C-cells in anterior segments (Fig. 4) was observed at somewhat later times in C-cells in the more posterior segments (Fig. 6). At E9 and E10, C-cells located posteriorly had fewer processes than those located anteriorly, and no C-cells had yet added the full complement. By E11 the more anterior C-cells had nearly their maximum number of processes, and by 12 days all of them had their full complement. The number of processes on all C-cells appeared to be a simple linear increase of about 21 processes per day—nearly one process per hour—from E9 to E11. (Because the number of processes on each cell reached a maximum, such a comparison is valid only before E12.) This rate of process addition was essentially identical in all body regions (the numbers of processes added during E9–E11 in the four regions were 42, 43, 41, and 39), justifying the conclusion that C-cells in different body regions develop in much the same way.

Therefore, we can estimate the anterior-to-posterior gradient of process addition during this time. Because each group of three or four segments differed from the adjacent group of segments in the same embryo by an average of 4.3 processes/C-cell (i.e., each segment was about 1.4 processes ahead of its posterior neighbor), and each cell added an average of 20.6 processes per 24 hr (i.e., it took nearly 1.2 hr to add each process), each segment in an embryo was about 1.6 hr ahead of its more posterior neighbor.

**Orientation of New Processes May Not Be Random**

The processes added at the ends of each C-cell were often not oriented obliquely, but instead were often oriented along the longitudinal (anterior–posterior) or circumferential (medial–lateral) axes (Figs. 3 and 5). These orthogonal axes corresponded to the rudimentary longitudinal and circular muscles already present when the C-cells were developing (Jellies and Kristan, 1988b; Jel-
Fig. 4. Growth of C-cells in segments 4-7. The average soma length in the anterior-posterior axis, normalized to the distance between segmentally homologous nephridiopores, is represented by the grey bars and the left axis; the average number of processes on each C-cell is represented by black bars and the right axis. Note that segmentally homologous C-cells encounter each other about 24 hr before having their complement of processes. Error bars represent ± SEM.

C-Cell Growth Cones Pause during Alignment and Then Advance Synchronously

In order to explain the rhomboidal shape of the older C-cells, in which all processes were approximately the same length (e.g., Figs. 1 and 10B), one must postulate either that each newly added process grows faster than the preceding one or that the older processes slow down considerably when they reach a particular length or position. Qualitative inspection of C-cells (Fig. 3) favors the idea that older processes slow their advance after becoming oriented obliquely. For instance, the longest growth cones during E10 were poised at the level of the ganglionic margin medially and at the nephridiopore laterally and they remained close to these landmarks 24 hr later; i.e., the processes grew at about the same rate as the germinal plate expanded. To quantify this observation we measured the medial-lateral distance between the ends of the longest processes at different developmental stages as a fraction of the distance from the ganglion to the nephridiopore (Fig. 8). This measure of relative growth normalizes for different-sized embryos, as well as for variations in pinning out the preparations. There was little relative growth of the processes until E12, when they began a clear increase in relative growth that followed the anterior-posterior gradient (Fig. 8). This growth spurt carried the growth cones beyond the ganglionic margin medially and the nephri-
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Fig. 6. Temporal progression of process addition in C-cells. Different shading on bars (inset) represents different groups of contiguous segments, with more posterior to the left at each time point. Measures are ±SEM.

diopore laterally, as seen in Fig. 1. We refer to the earlier period of no relative growth as the “pause” and to the later period of pronounced relative growth as the “rapid growth period.” This truly represents an increase in elongation rates of C-cell processes rather than a slowing of germinal plate expansion. The distance between the growth cone fronts as well as between the contralateral nephridiopores from E10 to E11 was expanding at about 3 μm/hr, whereas from E12 to E13 growth cone fronts were expanding at about 13 μm/hr and nephridiopores were expanding at about 6 μm/hour. These rates are, at best, crude estimates of growth since they do not account for individual variation. Neither are they intended to reflect actual migration rates since these measures do not take the oblique trajectory into account. Also, these rates are based upon distances between growth cone fronts moving away from each other rather than from a stationary origin (although, since expansion of the germinal plate and growth of the C-cell are symmetric, one might simply divide these figures by 2). However, they do illustrate that both the germinal plate and the C-cells increase their growth rates, but whereas the germinal plate between nephridiopores doubles in its rate of expansion, the C-cell growth cone fronts quadruple expansion rates during the same period.

These observations confirm that the older C-cell growth cones paused at a particular location in the developing body wall while additional processes were being added and aligned. Given the timing of the addition of processes (Fig. 6), the older processes paused for more than 72 hr (from E9 to E11), whereas the youngest paused no more than 24 hr before the rapid growth period started in all processes simultaneously. It is possible that the processes pause because they recognize par-
Fig. 9. The two locations at which outgrowing C-cell growth cones pause in the germinal plate. Shown is the boundary with two LY-filled C-cells abutting. The ends of their somata do not line up well with the long axis. The white triangle shows the end of the posterior cone, whereas the white arrows show the location of its most anterior growth cones. The process on the left is shorter than the one on the right, but both of their growth cones are roughly in line with all the other growth cones on their own side. Calibration = 50 μm.

Prior to E10, C-cells were less than one full segment in length (Fig. 3). When processes from adjacent C-cells on the same side met anteriorly and posteriorly (Fig. 10A), they initially intermingled. However, days later the processes of neighboring C-cells were aligned with the same orientation and spacing as adjacent processes of the same C-cell (Fig. 10B). Previously, we have shown that when one of these cells was killed by photoablation during E11 and examined later in development, the oblique muscles in that segment were completely missing and the oblique muscles in adjacent segments appeared to be completely normal (Jellies and Kristan, 1988b). This result implied that after E11, adjacent C-cells did not require an interaction with each other to maintain their trajectories or to project their growth cones accurately. To test this directly, we photoablated a single C-cell at E11 (see Materials and Methods) and then injected dye into the remaining C-cells on both sides of the lesion 72 hr later. In most cases (11 of the 12) C-cells adjacent to the lesion appeared normal. The single exception projected one errant process which is considered below. However, when single C-cells from the same segment were ablated at earlier stages (E9 and E10) and the embryos were allowed to develop for 72 hr, the C-cells adjacent to the lesion consistently invaded the vacant territory (Fig. 11), thereby narrowing the gap between the remaining segmental homologs (Fig. 11, large black arrows). There were also major errors in the trajectories of some of the processes of adjacent C-cells (Fig. 11, white arrows). Measurements of 24 C-cells in 12 animals (7 from E9, 5 from E10) showed that the invasion of the experimentally induced vacancy was greater when the ablation was performed on E9 than when it was performed on E10 (Fig. 12, black bars). However, there was no change in the number of processes in the adjacent C-cells on either day (Fig. 12, gray bars).

In all C-cells adjacent to an ablated C-cell, the spacing between the last four or five processes near the vacant territory appeared to be greater than normal. Measurements showed that the spacings in the experimentally induced vacancy were greater when the ablation was performed on E9 than when it was performed on E10 (Fig. 11, white arrows). There were also major errors in the trajectories of some of the processes in the adjacent C-cells. Spacings near the vacant territory were increased compared to the spacings in control, contralateral C-cells (Fig. 13) and that the spacings in the middle of the cell and at the opposite end were not different from control (Fig. 14).

The absence of one neighboring C-cell, therefore, had limited effects on the development of adjacent C-cells. However, such ablations removed only one of the two C-cell neighbors of a given C-cell. To determine whether interactions with the remaining C-cell on the side away from the ablation were responsible for maintaining this degree of normality in structure in the C-cells adjacent to the vacated territory, we removed both neighbors of a
given C-cell. Specifically, we photoablated C-cells from the same side in segment 6 and segment 8 at E9, leaving the C-cell in segment 7 with no adjacent C-cell neighbors. In all cases (9/9), the C-cell response to the double ablation was the same as the C-cell response to the single ablation: there was no change in the number of processes, and the somata extended about a quarter of the way into the vacated territory on both ends (Fig. 15).

**Interactions Restrict Navigation to Appropriate Trajectories**

In addition to filling in vacant territory, the most terminal processes of C-cells made major navigational errors, as shown in Fig. 11. Navigational errors were all of the same type: the errant processes grew along a trajectory perpendicular to the other processes of that cell (Fig. 11, white arrowheads), through territory not yet invaded by processes of any other C-cell. To determine the timing of these errors, single C-cells were ablated in embryos of different stages. When seven ablations were performed at E9, a total of 20 errors were found in the 14 C-cells adjacent to the lesions. Similarly, when five ablations were performed at E10 a total of 19 errors were induced in the 10 adjacent C-cells, but when six ablations were performed at E11 only 1 error was induced in the 12 adjacent C-cells. Furthermore, these navigational errors projected in reliable ways (Fig. 11): considering all major navigational errors, every one of the 21 errant processes on the posterior ends of C-cells projected posterior-lateral (away from the ventral midline), and every one of the 19 errant processes on the anterior ends of C-cells projected anterior-medial (toward the ventral midline). In other words, experimentally induced errant processes never crossed normally oriented processes of the same cell. In addition, the errant pro-
FIG. 11. Limited C-cell invasion of an experimentally created vacant territory. The ends of somata are indicated by large arrows, with the experimental side (right) compared to the control side (left). Projection errors are indicated by the white arrowheads. The spacing between adjacent processes becomes more normal as one moves away from the vacant territory. Calibration = 100 μm.
Fig. 12. Age-related effects of C-cell invasion of a vacant territory. The black bars are the measures of distance between ends of somata across a vacancy expressed as a percentage of the same measure on the control side (left axis); the grey bars are the number of processes (right axis). Error bars represent ±SEM.

processes were always the terminal ones, which were the youngest and, therefore, the most poorly oriented at the time of the ablations.

We were concerned that the aberrant projections were a nonspecific response to the manipulation which might have caused a general decline in vigor of the embryos. However, such a general deterioration is unlikely for two reasons. First, only the C-cells adjacent to the lesion were aberrant and only where they abutted the vacant territory, i.e., the other ends of the very same cells appeared normal (Figs. 11 and 14). Second, we found that the longest processes on C-cells adjacent to the lesion were the same length as their contralateral homologs: the difference at E9 was 5 ± 12%; the difference at E10 was 6 ± 6%; and the difference at E11 was 5 ± 9% (all ±SD). These results suggest that C-cells extend processes on a schedule and outgrowth rate that are independent of interactions with their neighboring C-cells, but that adjacent C-cells interact during E10 to properly space and align their processes at the border. The overlapping growth cones of adjacent C-cells extend long filopodia capable of a direct interaction (Fig. 10A).

Fig. 13. Average spacing between the last five processes adjacent to a vacancy. Interprocess distance was measured between the necks of adjacent growth cones and expressed as a percentage of the same measure on the control side. Error bars represent ±SEM.

Fig. 14. Transect measure of interprocess distance along a C-cell adjacent to vacant territory. Measures were made only from medial processes in segment 7 between normally oriented processes. Each interval was first normalized to its corresponding contralateral interval. The first spacing interval was that closest to the vacancy (inset), and all others were subsequently normalized to the first one. Therefore, in this plot, the ideal interval for normal, undisturbed processes would be about 0.5.

Fig. 15. Effect of isolating a single C-cell from both segmental homologs on E9. Measures are as in Fig. 12, showing the effect of ablat- ing either one (left) or two (right) C-cells. The left-hand bars are the same as presented in Fig. 12, repeated here to allow a direct comparison to illustrate that there was little or no effect of creating vacant territory on both sides of a C-cell. Error bars represent ±SEM.
FIG. 16. Transient dye-coupling among segmental C-cell homologs. Every other C-cell was filled with LY. At the top of each panel is the bottom edge of one C-cell filled with LY, and at the bottom of each panel is the top edge of a second LY-filled C-cell. In the middle is the space occupied by a C-cell in (A) segment 5 at E10, (B) segment 5 at E11, and (C) segment 16 in the same E11 animal shown in (B). White arrowheads indicate the C-cell that was not directly injected with LY (A, C). Note, particularly in (A), that some of the longitudinal muscle precursor cells also appear to be dye-coupled to the C-cells. Calibration = 100 μm.

One possible type of interaction is the formation of contacts large enough to allow passage of molecules, such as a fluorescent dye, from one cell to another (Taghert et al., 1982).

Segmental Homologs Exhibit Lucifer Yellow Coupling

To test whether the C-cells are dye-coupled during the time of overlap among their processes, we filled every other C-cell (N = 43) in six embryos with LY. We found that anterior C-cells were dye-coupled during E10, but there were no indications of such coupling before (E9) or after (E11) (Fig. 16). We did not determine whether the cells might remain electrically coupled at the stages when LY coupling could not be detected using the LY antibody (Taghert et al., 1982). Additionally, these cells become dye-coupled and then uncoupled in the same anterior-posterior fashion as seen for all other developmental features of this cell, as indicated by the fact that a C-cell in the posterior end of an E11 embryo was still dye-coupled (Fig. 16C) after the anterior C-cells had lost their dye-coupling. Interestingly, the termination of dye-coupling corresponds to the time when growth cones end their pause and begin their rapid growth phase. Notice also that there may have been some dye-coupling to longitudinal muscle founder cells (Fig. 16), but this finding was not entirely consistent and so was not analyzed here.

C-Cells May Die after Extending Processes Past the Dorsal Midline

At E9, the embryo consists of a germinal plate, a relatively flat sheet which expands both longitudinally and laterally over the surface of a larval sac (Fernandez and Stent, 1982). Ultimately, the lateral edges meet and fuse, thereby forming the dorsal midline. This fusion begins at the anterior end of the animal at about E17.
embryos were filled with HRP in living and undissected animals. Three successful fills in different preparations were obtained and the animals were dissected along the ventral midline, opposite to the orientation of all other dissections, in order to visualize the dorsal midline. In all three cases, the C-cell growth cones from segment 2 had indeed crossed the fused midline and continued on in a correct trajectory for a short distance (not shown). Although growth cones in culture can survive for an extended period when severed from the cell body (Guthrie et al., 1989; Shaw and Bray, 1977), we have not addressed that possibility in this system.

DISCUSSION

Summary of C-Cell Development

This study examined the morphogenesis of the C-cell, an identified cell with a spindle-shaped soma and multi-
ple parallel processes extended in a regular array. Although the C-cell has a very regular final form, its development suggests that its stereotypy emerges as the result of several qualitatively different developmental events:

1. The first differentiated C-cell appears sometime before E7 of embryogenesis from an as yet unidentified precursor. When first identifiable, the C-cell has only a few processes and lies in the stereotyped location in which it can be found throughout the remainder of embryogenesis. At these earliest stages, the outline of the C-cell is ovoid and there is a gap between segmentally homologous C-cells.

2. The C-cell adds processes for 4-5 days of development, primarily at the anterior and the posterior ends, and thus becomes progressively elongated. When processes first arise they are not oriented obliquely; rather, they commonly project parallel to previously formed longitudinal and circular muscle cells. These processes grow in absolute length and take an orientation oblique which it can be found throughout the remainder of embryogenesis. At these earliest stages, the outline of the C-cell is ovoid and there is a gap between segmentally homologous C-cells.

3. After this initial orientation, the terminals of the processes grow slowly, just fast enough to keep their growth cones at particular locations in the body wall for an extended period. This slow phase of extension continues until the cell has added its entire complement of processes. There is little or no elongation of C-cell processes relative to the growth of the germinal plate during this period.

4. The number of processes on each C-cell is fixed at about 70; so far, no manipulation has succeeded in changing this number.

5. Near the end of the period of adding processes, filopodia at the anterior and the posterior ends of segmentally homologous C-cells intermingle and adjacent C-cells become transiently dye-coupled (as revealed by LY transfer). While dye-coupled, the newly added processes in adjacent C-cells orient obliquely and parallel to each other and then the cells dye-uncouple. (As a caveat, it is possible that adjacent C-cells remain coupled but do not pass sufficient dye to be detected by our techniques.)

6. C-cell processes then undergo a rapid growth phase in which they elongate relative to expansion of the germinal plate for about 8 days. During this time, their growth cones advance as a unit, with their filopodia within easy reach of one another, and the processes maintain their oblique trajectory. During this period of relatively rapid elongation, definitive myocytes collect along each extending C-cell process to form the oblique muscle layer (Jellies and Kristan, 1988b).

7. As the C-cell growth cones approach the fusing edges of the germinal plate (the future dorsal midline), the C-cell somata appear to degenerate and probably die at about the time that the growth cones cross the fusing dorsal midline. (We have not addressed the possibility that C-cell growth cones, separated from their soma, are capable of continuing their spiral trajectories across the dorsal midline and track along the established oblique muscle fascicles.)

From the errors seen in normal development and in response to ablations of muscle precursors (Jellies and Kristan, 1988c) and other C-cells (Figs. 12-15), we conclude that the processes of C-cells: (1) strongly prefer to orient obliquely, i.e., the only orientations of long processes are 45° or 135° to the longitudinal axis; (2) strongly prefer growing anterolaterally if they are on the lateral side of the soma and posteromedially if they are on the medial side; (3) will sometimes grow postero-laterally if they are on the lateral side and anteromedially if they are on the medial side; and (4) never grow laterally if they start on the medial side nor medially if they start on the lateral side. The remainder of this section discusses the successive stages of development and proposes a mechanism which could produce both normal embryogenesis of the C-cell processes and their responses to ablations.

**Early Addition and Orientation of Processes**

During the early phases of C-cell growth, newly forming processes appear to grow along cells which are the precursors of the longitudinal and the circular muscles. This association must be quite transient, however, because only the newly added ones exhibit such orthogonal (as opposed to oblique) orientations (Fig. 7). The C-cell growth cones may receive initial orientation cues from these early muscle cells, a mechanism widely found in the early guidance of neuronal growth cones, both within the central nervous system (Bastiani and Goodman, 1986; Ghosh et al., 1990; Goodman et al., 1984, 1984; Kuwada, 1986; Kuwada et al., 1990; Stainier and Gilbert, 1990; Taghert et al., 1982; Wilson et al., 1990) and in the periphery (Bate, 1976; Bentley and Keshishian, 1982; Eisen, 1988; Ho and Goodman, 1982; Jellies and Kristan, 1988a; Kuwada, 1986; Tosney, 1988, 1991; Tosney and Landmesser, 1985). If the growth cones have equal affinity for the circular and the longitudinal fibers, for instance, the processes might tend to grow halfway between, i.e., in an oblique direction. It is also possible, however, that the circular and the longitudinal muscle precursors are simply the first permissive cellular sub-
strates encountered and that they provide no orientation cues or even wrong cues that must be overcome to grow properly.

An attempt was made to determine the importance of these muscle precursors by photoabrupting particular ones during El0-El1 (Jellies and Kristan, 1988c). These ablations produced a significant increase in the number of navigational errors of the C-cell processes, but the total number of errors was still very small. This small effect of ablating the muscle precursors could mean that these cells are relatively unimportant for setting up the pattern of C-cell processes. Alternatively, the muscle precursors may have a major, but indirect, role. For instance, these early muscle cells may establish an orthogonal grid which they imprint on other cells or extracellular matrices so that removal of the precursors themselves at El0 or later has little effect on the orientation of C-cell processes. Another possibility is that there are multiple influences on the outgrowing processes and that removal of any one of them has little effect (Blair and Palka, 1985; Caudy and Bentley, 1986a,b; Landmesser et al., 1988). To determine whether either of these possibilities explains the results of these photoablations, it will be necessary to ablate the muscle precursors in a variety of combinations at earlier times in development. Once the orientation of the first couple of adjacent processes are established, they may interact with newly forming processes to orient them properly. If continued, this mechanism could, like falling dominos, properly orient all the processes, whether or not the muscle precursors continue to be present.

One Possible Mechanism for Orienting C-Cell Processes: Avoidance among Adjacent Growth Cones

What sort of interaction might be responsible for orienting and spacing the outgrowth of new processes? One possibility is an “overgrowth-and-paring-down” model in which new processes are added at all possible orientations and those oriented improperly or having the wrong spacing are retracted or removed. Although our data do not rule out this mechanism, new C-cell processes do not appear to be randomly arranged, neither are there degenerating processes just back from the ends of the somata, as would be expected of an overgrowth-and-paring-down mechanism. Instead, we think it is more likely that the newly added processes initially orient along circular or longitudinal muscle precursors (Fig. 7) and are secondarily oriented obliquely so that they come to lie at an angle to these orthogonal muscle layers. A mechanism that might help to produce this oblique orientation is suggested by the avoidance interactions among neighboring C-cells (Fig. 10): growth cones may repel one another possibly by each one inhibiting the growth of the other when their filopodia are in contact (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987; Moorman and Hume, 1990). Figure 19 shows how such inhibitory interactions might help to promote C-cell development, as well as account for the errors seen in response to ablation of neighboring C-cells.

Normal C-cell development. Figure 19A represents the endpoint of the slow phase of process outgrowth, with all processes present and oriented properly. It also indicates the sites of inhibitory interactions among all the processes. Figure 19B represents an earlier stage in the cell's development, with the middle processes oriented properly but with the terminal processes oriented orthogonally along muscle precursors. The processes labeled w-z are all improperly oriented and represent the major problems faced by C-cell processes in becoming properly oriented. The newly emerging process W has lined up with circular muscle precursors; it could be properly oriented by being pushed toward the end of the soma by inhibitory interactions with already-existing processes. Process X is also lined up with circular muscle precursors, it can be pushed into its proper oblique orientation by inhibitory interactions with newly forming processes being added at the growing tip of the soma. Processes Y and Z are lined up with longitudinal muscle precursors; they also could be pushed into place by crowding from newly emerging, repellant processes being added medially to them at the somatic tip. This
somewhat swing of processes Y and Z would stop only when they came under the inhibitory influence of the already-existing processes, which themselves are properly oriented.

**Errors in orientation.** Most processes at the ends of mature C-cells facing the open spaces produced by photoablation orient properly but have larger spacings than normal between them (Figs. 11, 13, and 19C). To an extent, the proposed inhibitory interactions can account for this result: the processes at the end of this C-cell lack inhibition from the processes of their segmental neighbor (which has been ablated), so they can more fully express the effects of the inhibition from their own adjacent processes and stay further from them. This explanation cannot fully explain the results, however, because if only repulsion between adjacent growth cones were responsible for the orderly orientation of processes (Figs. 1 and 10), the processes at the end of a C-cell growing into vacant territory should fan out as they grow to maximize the spacing between them. Instead, most of the C-cell processes entering vacant territory remained parallel to the correct orientation (Figs. 11 and 19C). These observations suggest that there are directional cues in the vacated territory, and these cues are the major influence causing the processes to orient obliquely. In support of this is the observation that those few processes growing into vacated territory in other than the proper orientation did so along the oblique paths normally taken by C-cell processes from the opposite side of the embryo, implying that there are two preferred trajectories—mirror images of one another—guiding the growth of C-cell processes in any segment.

Given the observations that the number of processes produced by a C-cell appears to be determined by inherent mechanisms (or, at least, by mechanisms resistant to any perturbations so far produced) and that the major control of process orientation can be accomplished without close contact among processes, what then is the function of inhibitory interactions among processes? We propose that such interactions are important for packing the fixed number of processes into the available space at relatively equal intervals. If there is more space the processes do not pack so densely, but, once packed, the processes retain their tight packing. A second function of these inhibitory interactions may serve to nudge the processes into the correct trajectory of the two possible ones. For processes like W and X in Fig. 19, this involves a movement of 45° or less, through angles not permitted by the hypothesized directional cues. Processes Y and Z, however, would have to be pushed through nearly 135°, past an orientation (at about 45°) that is permissible. It appears, in fact, that most processes which grow at the incorrect angle into vacated territory are just such processes (Figs. 11 and 19C): those which might have gotten hung up in the middle of being pushed into the proper orientation. The fact that such errors are found only at the ends of the C-cells may be because these processes lack guidance at a critical time in their development: the C-cell has stopped adding processes to help orient them, and the neighboring C-cell processes have been ablated. A third function of these inhibitory interactions may be to recover the spacing between processes that have separated, either by accident or after growing around structures in the body wall such as the nephriodiopores (e.g., Fig. 1).

We do not know how the avoidance is mediated, if actual contact (and coupling) is required or if cells could be responding at some distance to a diffusible substance produced and released from all C-cells. Indeed, processes move into correct orientations for several days before segmental homologs contact one another.

An alternative to this inhibitory interaction among processes is that each process has an intrinsic polarity, perhaps involving the cytoskeleton, that restricts it to one oblique orientation. Our experiments do not address this issue, but it is clear that any intrinsic mechanism cannot be the exclusive controller of orientation or the ablation of neighbors would not produce the observed effect on orientation. It would not be surprising, in fact, if intrinsic mechanisms turn out to help in producing the orientation, since the production and maintenance of such a complex and organized structure is likely to be regulated by a number of factors.

**Possible Function for Coupling between Segmentally Adjacent C-Cells**

If there is a strong inhibitory interaction among C-cell processes, how can the processes of contralateral homologs cross each other at about 90°, apparently without pause? Two possibilities are that the two sides have different guidance cues—e.g., the two mirror-imaged oblique trajectories have different markers on them—or that the contralateral C-cell homologs respond differently to the same sets of cues. If so, it is hard to explain that errant processes do not grow randomly but instead follow the paths that are normally followed by processes of the contralateral homolog (Fig. 11). One explanation is that only processes of the same cell normally inhibit one another and that one of the functions of the direct coupling observed among segmental (ipsilateral) homologs is the conferring of self-recognition on neighboring C-cells, so that thereafter the growth cones from once-coupled cells repel each other. In this scheme, the events of recognition, repulsion, and directional guidance are related to, but distinct from, one another since they are separable by experimental ma-

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nipulation. This might also help explain the different phenomenology of dye-coupling among regenerating versus initially developing S-cell neurites (see review by Muller, 1988).

The mechanisms that underlie self-recognition are obscure, but coupling and junctional communication have long been implicated in this and other patterning events (Fraser et al., 1988; Gilula, 1985; Lo, 1988; Revel et al., 1985; van den Biggaelaar and Serras, 1988). If such a recognition event were not available to contralateral homologs in our system (contralateral homologs show no overt dye-coupling using our techniques, but see caveat presented previously), then they would not repel one another. This idea is potentially testable in that one might predict that if contralateral homologs could be brought into contact earlier in development and made (or allowed) to couple, then they might show repulsion similar to that seen between growth cones of ipsilaterally homologous C-cells. Although this speculation is consistent with our observations, we currently have no evidence to support or refute it.

C-Cell Growth Cones Pause for an Extended Period

During initial outgrowth, the C-cell growth cones effectively pause for an extended period at a particular location in the body wall. This has the effect of aligning the fronts of growth cones and coordinating the extension of C-cell processes with the rapid expansion of the germinal plate. Such pauses during growth cone migration are suggested in many instances in both vertebrate (Bovolenta and Mason, 1987; Godement et al., 1990; Myers et al., 1986; Tosney and Landmesser, 1985) and invertebrate (Caudy and Bentley, 1986a; Kuwada, 1985) development, where they seem to be associated with choice points in the environment. Different rates of extension also may indicate different modes of growth cone extension (O'Connor et al., 1990). This slow growth phase may reflect a period in which the C-cell is committing much of its resources to aligning its processes properly and very little to growth of the existing processes.

Later Process Elongation

C-cell growth cones continue to extend obliquely and parallel to one another at least until the edges of the germinal plate fuse at the dorsal midline. During this time (E12–E20) the germinal plate, and consequently the environment through which the growth cones navigate, is rapidly expanding (Fernandez and Stent, 1982). If these processes were growing along some sort of preset guidance tracks, the tracks would have to be reformed continually because cells and matrix appear to be added both at the edges of the germinal plate and interstitially along the entire plate. Observations of errors in trajectory (Jellies and Kristan, 1988d) also seem inconsistent with discrete guidance tracts, since errant growth cones can insinuate themselves between existing ones and single or small numbers of growth cones can migrate along an oblique trajectory well in advance of other processes (Fig. 11). Thus, these growth cones would be encountering an environment that is spatially and temporally mismatched in local cues, yet they project along an oblique trajectory consistent with their ability to utilize more global cues, such as gradients of morphogens or cells (Harris and Holt, 1990; Marchase, 1977; Nardi, 1983; Trisler, 1987).

We are currently examining the growth cones, processes, and environment at the EM level. Our initial observations fail to reveal any discrete compartmentalization or potential guidance cues of an anatomically simple and discrete nature (Kopp, McCarthy, and Jellies, unpublished). We therefore favor, as a working hypothesis, the idea that the continued growth and navigation of these growth cones, like the early growth and orientation, are directed by multiple cues that are integrated by the growth cones.

C-Cell Degeneration

Although no C-cells have been found after 20 days, even when filled with HRP at 18 days, it remains a formal possibility that they are present. We feel, however, that our present results are more consistent with the idea that these cells are degenerating over the course of several days and that our failure to find them is because they have died. Attempts to identify dead C-cells by selective dye uptake have so far proven negative (Jellies, unpublished). In fact, C-cell death at about 20 days might be appropriate, because the grid of oblique myocytes has been established over the entire body by then (Jellies and Kristan, 1988b) so that the scaffold of C-cell processes seems no longer necessary.

The C-cell in the medicinal leech presents us with a particularly unique experimental situation. The preparation offers the advantages associated with the identifiable character of simplified systems while retaining a diversity of complex features that make it likely that this system utilizes multiple guidance mechanisms. The results presented here suggest that, as in many vertebrate systems, the stereotyped navigation of growth cones of this identified cell in the leech appears to depend on spatial and temporal interactions between qualitatively different developmental mechanisms, both intrinsic and extrinsic. Because it is possible to manipulate the environment of the outgrowing processes of this cell within the embryo in very precise ways, it is a particularly favorable preparation in which to examine how
complex patterns emerge during normal development in an intact developing embryo.

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REFERENCES


Harris, W. A., and Holt, C. E. (1986). Early events in the embryogen-


