Cell Recognition by Neuronal Growth Cones in a Simple Vertebrate Embryo

JOHN Y. KUWADA

The mechanism that guides neuronal growth cones to their targets in vertebrate embryos has been difficult to study primarily because of the complexity and large number of neurons found in many vertebrate nervous systems. The spinal cord of a simple vertebrate, the fish embryo, is used to analyze pathfinding mechanisms. The early embryonic spinal cord consists of a relatively small number of identifiable neurons. From the beginning of axonal outgrowth the growth cones of these identified neurons extend along stereotyped and precise pathways in the spinal cord. Laser ablation experiments (i) support the hypothesis that early growth cones that pioneer specific spinal tracts appear to recognize cues on subsets of longitudinally arrayed neuroepithelial cells and (ii) demonstrate that later growth cones that selectively fasciculate in these spinal tracts appear to recognize cues on specific subsets of axons.

WITHIN THE CENTRAL NERVOUS SYSTEM (CNS), INDIVIDUAL neurons are interconnected in a remarkably specific way. Understanding how these specific connections are formed during development involves, in part, elucidating the mechanisms that guide growing neurons to their correct targets. Since the pioneering work of Ramón y Cajal and Harrison at the turn of the century (*1*), much emphasis has been focused on the important role of the growth cone, the amoeboid-like motile extension at the tip of the growing axon.

Yet only recently have investigators begun to characterize in detail the behavior of individual growth cones during embryonic development, and to experimentally analyze the mechanisms that guide these growth cones to their correct targets. For example, in the developing CNS of vertebrates, numerous studies have demonstrated that the early growth cones that pioneer specific pathways extend along nonneuronal cells such as neuroepithelial or primitive glial cells. Such observations led to the proposal of the "blueprint hypothesis" of neuroepithelial pathways (*2*), and to the general notion that preformed glial or epithelial pathways throughout the CNS help to guide pioneering growth cones (*3*). During the development of neuromuscular specificity in the chick hindlimb, experimental analysis of growth cones of spinal motoneurons has implicated the important role played by local nonneuronal cues at the base and within the limb bud (*4*).

The mechanisms of growth cone guidance have been most thoroughly investigated in the relatively simple developing CNS of the grasshopper embryo (*5*). These neurons are identifiable on a number of criteria including their cell body size, location, and individual morphology. Descriptive analysis has shown that growth cones reach choice points where they contact several different axonal pathways, ultimately making specific choices as they selectively fasciculate with a specific subset of axons. Extensive experimental analysis supports the "labeled pathways hypothesis," which predicts that neuronal growth cones can recognize specifically labeled axonal pathways. More recent evidence in this system has implicated a role for identified nonneuronal cells in the initial establishment of axonal pathways (*6*).

Do the same kinds of specific neuron-neuron recognition occur during vertebrate development as occurs in the grasshopper? Moreover, what are the relative roles of nonneuronal and neuronal cues in the guidance of individual growth cones in the developing vertebrate spinal cord? These questions can be answered in the relatively simple and highly accessible spinal cord of the fish embryo. Recent investigations of the hindbrain and spinal cord of the fish have shown that many of its neurons can be identified as either specific individuals or members of a defined class (*7*). Since some fish embryos are exceptionally transparent, their individual embryonic neurons can be visualized with Nomarski optics, penetrated with intracellular microelectrodes, and filled with various intracellular dyes. Furthermore, a laser microbeam can be focused through the intact embryo onto individual cells for specific ablation experiments.

These techniques can be used to analyze the guidance of identified growth cones in the developing vertebrate spinal cord with the same sort of detail previously possible only with insect embryos. Here I use the Japanese medaka (*Oryzias latipes*) (*8*) to demonstrate that the growth cones of fish spinal neurons extend along stereotyped pathways in the embryonic cord from the beginning of axonogenesis. Laser ablation of specific neurons suggests that growth cones are guided to their targets by recognition of specific cellular cues.

Identified growth cones in the developing fish spinal cord. The neural keel of the fish embryo, which develops into the spinal cord, is initially composed of neuroepithelial cells that give rise to both neurons and glia. The embryonic spinal cord (Fig. 1A) is delimited at its outer surface by a basal lamina; just adjacent to this basal lamina are the endfeet processes of the neuroepithelial cells (Figs. 2 and 3). The neuroepithelial cells and their corresponding endfeet processes are found layered dorsal to ventral in the early cord. The developing spinal cord becomes filled with neuronal cell bodies, and many of these neurons extend axons in discrete fascicles that form in the outer margin of the cord adjacent to the neuroepithelial endfeet (Figs. 1A, 2, and 3). Many of the earliest neurons in the fish spinal cord can be identified (*9*), including both the primary axial motoneurons (*10*) and the primary mechanosensory neurons, called Rohon-Beard (RB) cells (*11*). The RB neurons are large,

The author is in the Department of Biological Sciences, Stanford University, Stanford, CA 94305.

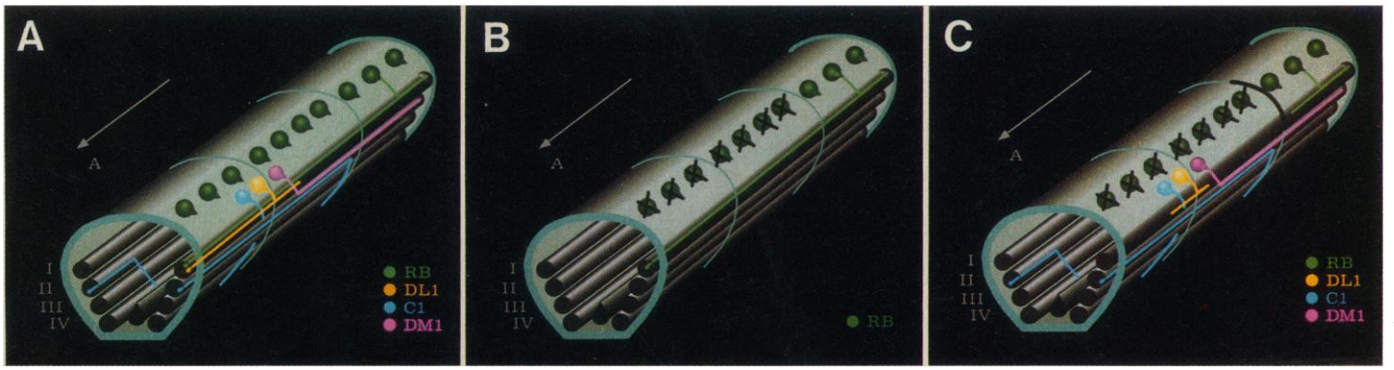

Fig. 1. Schematic representation of the general morphology of several segments of the spinal cord and identifiable neurons from relatively mature (100-hour) fish embryos. The gray region outlining the perimeter of the cord denotes the basal lamina and the endfeet processes of neuroepithelial cells. The gray cylinders represent longitudinal tracts. The precise number of tracts is not known. (A) The RB, DL1, DM1, and C1 neurons from normal embryos. Three cord segments are shown. (B) The RB neurons from a control segment just posterior to segments in which the RB neurons had been ablated with a laser microbeam in ovo at 40 hours of development. (C) The RB, DL1, DM1, and C1 neurons from an embryo in which the RB neurons from a number of segments had been laser-ablated and the dorsolateral region of those segments had been isolated from the adjacent control segments to prevent RB axons from those flanking segments from crossing into the experimental segments. The arrow and **A** denote anterior.

conspicuous cells (10 to 20 μm) found near the dorsal surface of the spinal cord of relatively mature embryos (Figs. 1A and 4A). Each spinal segment (as defined by the metameric arrangement of axial muscles) contains between one to four RB neurons on each side of the midline (that is, one to four per hemisegment). Each RB neuron has central axons that extend longitudinally in the dorsal most longitudinal tract, called the dorsolateral fascicle (DLF) (Figs. 1A and 2), and a peripheral axon that innervates the skin (*12*).

Many spinal interneurons can also be identified in medaka embryos. For example, in addition to RB neurons, the dorsal portion of each hemisegment contains at least three other interneurons called DL1, DM1, and C1 (Figs. 1A, 4B, and 5C). There are probably one of each per hemisegment. Their cell bodies are adjacent to each other, often near the segment border, and ventral to the RB cell bodies. The DL1 neuron has a short posterior axon (less than one segment long) and a long, branched anterior axon (which extends for four to nine segments), both of which run in the DLF. Interestingly, the DL1 axons appear to fasciculate with the axons of the RB neurons (Fig. 2). The DM1 neuron has only a posterior axon (greater than four segments in length) found in a specific longitudinal tract that lies ventral to the DLF (Figs. 1A and 5C). The C1 neuron is a commissural interneuron whose axon crosses the midline circumferentially at an oblique angle and then extends anteriorly (more than four segments in length) in the longitudinal tract just ventral to the DLF (Figs. 1A, 2A, and 5C).

The pattern of axonal outgrowth by the RB neurons is highly stereotyped from the start (Fig. 6). The RB neurons are initially located in the dorsolateral region of the developing spinal cord and are among the first neurons to project axons. They extend two growth cones, one anteriorly and the other posteriorly, at about 40 hours of development. By 56 hours of development the RB axons are up to ten segments long in each direction and the peripheral axon has branched from the central longitudinal axon. In 100-hour embryos, RB axons (four to eight segments) do not extend as far as in 56-hour embryos and the posterior axon is often shorter than the

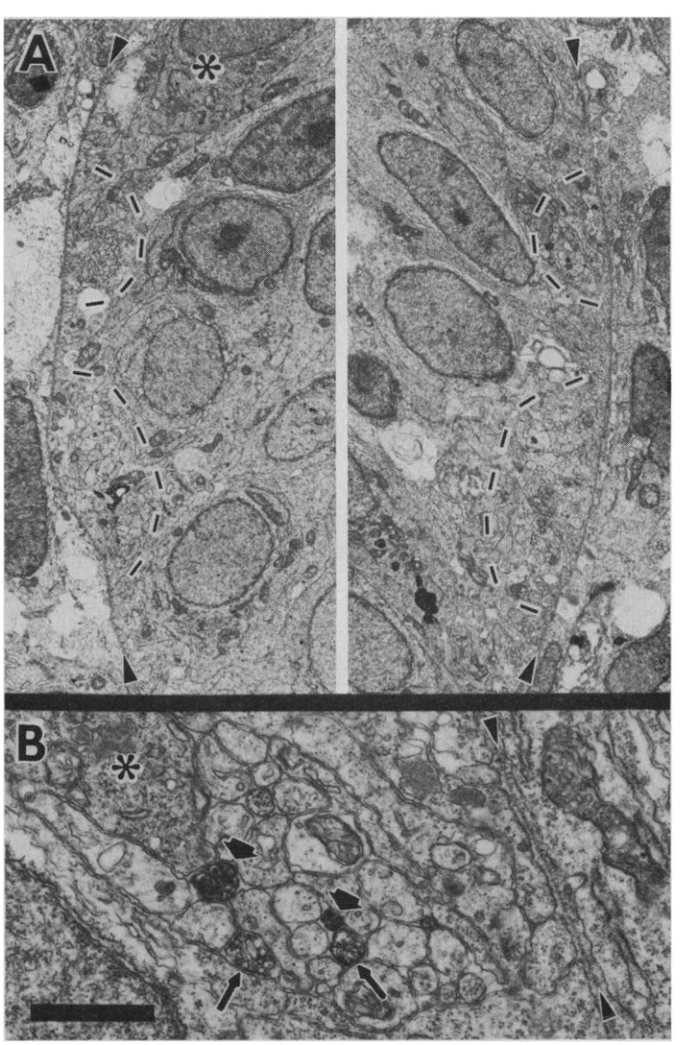

Fig. 2. Longitudinal tracts in the early spinal cord. (A) Electron micrographs of the left and right dorsolateral halves of the spinal cord from a 60-hour embryo cut in cross section. The DLF (upper) and the next more ventral longitudinal tract (lower) are outlined by the dashed lines. Other longitudinal tracts ventral to these are found in the ventrolateral halves of the cord, which are not shown. Parts of the stained growth cone of a C1 neuron filled with horseradish peroxidase can be seen in the lower tract on the left side. Horseradish peroxidase–stained axons of an RB and a DL1 neuron are visible in the DLF on the right side. The basal lamina that surrounds the embryonic cord is the dark staining material in between the arrowheads. The asterisk marks an RB cell body. (B) Electron micrograph at a higher magnification of part of the right DLF from (A) showing the darkly stained axon branches of an RB neuron (large arrows) and the lightly stained axon branches of a DL1 neuron (small arrows). Scale (A), 5 μm; (B), 1 μm.

anterior one (Fig. 5B). In 120-hour embryos the posterior axon of RB neurons has regressed with the axon less than a single segment in length in the most extreme cases (Fig. 6). The anterior axon is typically three to four segments long. Furthermore, the RB neuron is now monopolar, the cell body having assumed a position much closer to the dorsal midline, and the anterior axon is highly branched.

The RB growth cones pioneer the DLF. Reconstructions (n = 3) of electron microscope serial sections (*13*) demonstrate that early in development the only axons and growth cones found in the DLF all belong to RB neurons (Fig. 3). During these early stages the RB growth cones contact two substrates: (i) the axons and cell bodies of other RB neurons, and (ii) the endfeet processes of the dorsal neuroepithelial cells which are the only cells found in the dorsolateral portion of the cord at this time (Fig. 3). The endfeet processes of dorsal neuroepithelial cells run longitudinally for approximately 10 to 20 μm at this stage and are restricted to the dorsolateral region of the cord. Likewise, the endfeet processes of more ventral neuroepithelial cells are found only in the more ventrolateral regions (Fig. 7). The RB neurons are not mechanically restricted to the dorsolateral longitudinal region of the cord, since they project filopodia ventrally for more than 5 μm (Fig. 3B). Such filopodia are long enough to contact the endfeet processes of neuroepithelial cells 5 μm ventral to the DLF where the next longitudinal tract forms later (Fig. 2A).

Neurons that project their growth cones into already established tracts also navigate through the cord in a stereotyped and precise pattern. For example, the DL1 interneuron projects two growth cones at 56 hours in development into the DLF, 16 hours after the RB growth cones. Over the next 50 hours the two growth cones extend asymmetrically; the anterior growth cone extends anteriorly for four to nine segments while the posterior growth cone extends less than one segment (Fig. 4B).

Pioneering growth cones may use specific neuroepithelial cues. As described above, the RB growth cones pioneer the DLF. In so doing, they contact both other RB neurons and the endfeet of dorsal neuroepithelial cells. It seems likely that one or both of these substrates might provide their major guidance cue. To examine the possible role of neuronal surfaces (that is, contact with neighboring RB neurons), I examined the behavior of RB growth cones as they grew into neighboring spinal segments that were devoid of other RB neurons. To carry out this experiment, a laser microbeam was used to ablate RB cell bodies in vivo (*14*) on one side from three to six segments in embryos at a stage (40-hour) when the RB neurons are initially projecting growth cones. Remnants of the laser-ablated RB neurons were quickly removed since light micrographs of serial thick sections and electron micrographs of these embryos (n = 3) showed that 3 hours after the ablations no degeneration products were evident. Furthermore, the dorsal neuroepithelial cells and their endfeet processes appeared normal, an indication that these ablations were highly specific (Fig. 8, A and B). Electron micrographs of embryos (n = 3) 60 hours after ablations of RB neurons reinforced this by revealing no evidence of general damage to the cord, and no specific damage to individual neurons. Moreover, axons extended into the dorsolateral region where the RB neurons had been ablated, and the neuroepithelial endfeet in that region appeared normal (Fig. 8C).

In such embryos the growth cones of RB neurons from segments adjacent to the experimental segments were able to traverse the experimental segments along their normal dorsolateral pathway at

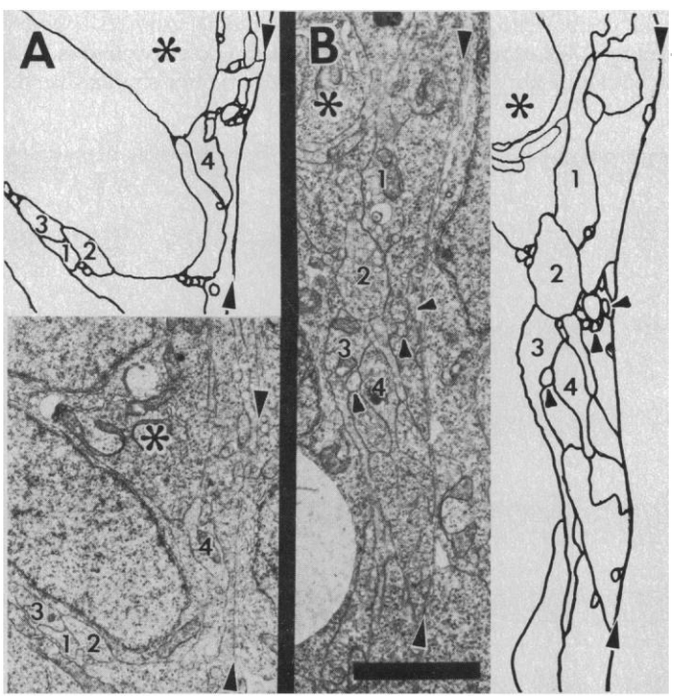

Fig. 3. The RB neurons pioneer the DLF. (A) An electron micrograph and drawing of the micrograph from the dorsolateral region of a 43-hour spinal cord showing an RB growth cone labeled **4** in contact with the cell body of another RB neuron (asterisk) and the endfeet processes of dorsal neuroepithelial cells. Profiles labeled **1**, **2**, and **3** are axons of yet other RB neurons. Nearly all the profiles adjacent to growth cone **4** that are not labeled are endfeet processes. In these sections, processes were identified by following them in serial sections to their cell bodies. The size and location of the cell bodies were used to identify a cell as an RB neuron or a neuroepithelial cell. The basal lamina is denoted by the large arrowheads. Dorsal is up and medial is to the left. (B) An electron micrograph from a section approximately 20 μm anterior to the section in (A) showing the growth cone of the RB neuron labeled **2** and its filopodia (small arrow heads) in contact with other RB axons and neuroepithelial endfeet processes. In addition, a long ventrally and circumferentially directed filopodium projected from the RB labeled **3** can be seen. The asterisk marks a different RB cell body from the one shown in (A). Again, nearly all the unlabeled profiles in contact with growth cone **2** are endfeet processes of neuroepithelial cells different from the ones shown in (A). The numbers identify the same RB neurons in both sections. Scale (for both panels), 2.5 μm.

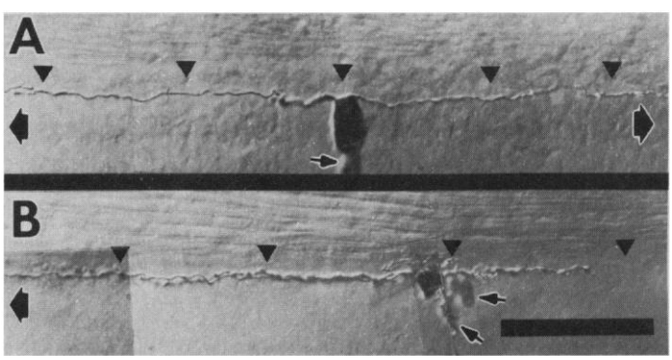

Fig. 4. The morphology of identifiable spinal cord neurons in fish embryos. (A) A dorsal view of an RB neuron from a 74-hour embryo that had been filled with Lucifer yellow dye and treated with an antiserum to Lucifer yellow and a horseradish peroxidase–conjugated secondary antibody in order to visualize it with Nomarski optics. The longitudinal RB axon runs along the lateral border of the cord. The anterior and posterior axons were ten and five segments long, respectively. The metameric, axial muscles are above the RB axon. Anterior is to the left in this and all subsequent figures with the same perspective. Triangles denote the approximate segment borders and large arrows indicate that the axon extends beyond the limits of what is shown. Small arrows mark neuroepithelial cells that are often filled with dye as the dye-filled microelectrode passes through them. (B) A dorsal view of a DL1 neuron from a 100-hour embryo. The DL1 anterior axon was five segments long. Scale (both panels), 50 μm.

both 56 hours (n = 8) and 100 hours (n = 5) of development (Figs. 1B and 9A). These results demonstrate that RB growth cones do not require neighboring RB neurons in order to pioneer the DLF; it appears as if neuronal surfaces are not necessary for establishing this longitudinal tract. Rather, given that RB growth cones normally extend in close contact with neuroepithelial endfeet, these results are consistent with the hypothesis that the neuroepithelial cells play a major role in their guidance.

Follower growth cones use specific axonal cues. Most later growth cones in the spinal cord choose among an array of already established axonal tracts. To examine how these follower growth cones choose their pathways, pathfinding by the DL1 neuron was analyzed. The DL1 growth cone is projected into the DLF 16 hours after it is pioneered by the RB neurons, and the DL1 axon fasciculates with the RB axons (see above). By approximately 100 hours, the DL1 normally has an anterior axon four to nine segments in length (Fig. 4B).

The role of the RB axons in the guidance of the DL1 growth cones was investigated by specific laser ablation experiments. In control experiments, RB neurons were ablated (just as described above) from one side of a number of segments in 40-hour embryos. Sixty hours later, intracellular dye injections showed that the flanking RB axons had extended through the experimental segments (described above), and that the DL1 neurons within those experi-

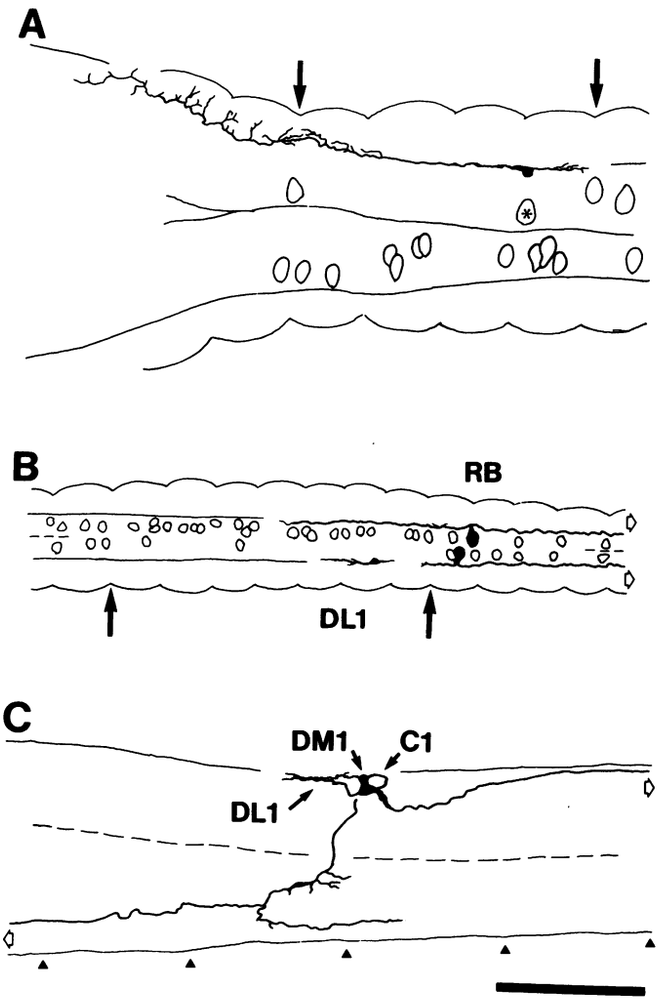

Fig. 5. The efficacy and specificity of the laser ablation technique. (A) A camera lucida drawing of a dorsal view of a cord from a 100-hour embryo in which RB neurons were ablated (between the arrows) on one side 60 hours earlier. The dorsolateral portions of these experimental segments were not isolated from adjacent segments. The black cell is a DL1 neuron from an experimental segment. The cell bodies of nearby RB neurons are outlined. The RB neuron denoted with an asterisk had no axons. Segments are indicated by the axial muscle groups on either side of the cord. (B) Dorsal view of the cord from a 100-hour embryo in which the RB neurons were ablated (between the arrows) and the dorsal lateral regions of the experimental side of these segments were isolated from the adjacent control segments. Drawn are an experimental DL1 and RB neurons from an unmanipulated segment. Arrows denote that the axons extend beyond the limits of the drawing. (C) The DL1, DM1, and C1 neurons from the experimental side and segment from a 100-hour embryo that received the ablation and isolation procedure. RB neurons were ablated on the experimental side in segments 6 through 10. The neurons shown are at the border of segments 7 and 8. Scale (A), 120 μm; (B), 200 μm; (C), 50 μm.

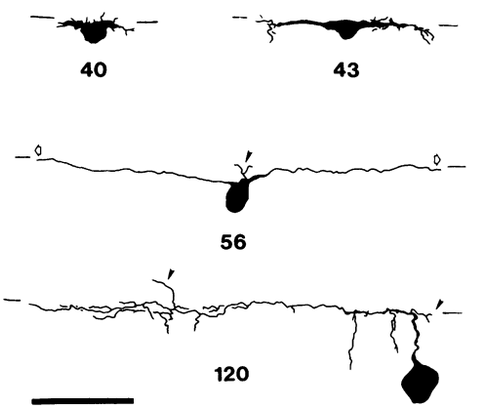

Fig. 6. The morphological development of the RB neuron. Camera lucida drawings of RB neurons from a dorsal perspective from embryos at different stages of development. The numbers below each drawing designate the age in hours of the embryos. For each stage at least eight neurons were filled. The axons of the 43-hour RB were one segment long each; the anterior and posterior axons of the 56-hour RB were nine and seven segments long, respectively; the anterior axon of the 120-hour RB was three segments long. Horizontal lines denote the lateral edge of the cord and anterior is to the left. Arrows indicate that the axons extend beyond the drawings and the arrowheads point to the peripheral axons of which only their proximal-most parts are drawn. Scale, 50 μm.

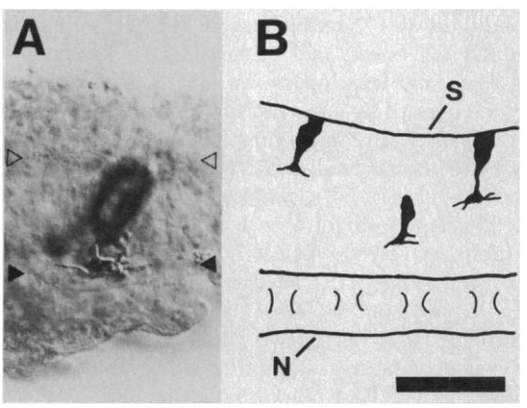

Fig. 7. The morphology of neuroepithelial cells in the spinal cord of 40-hour embryos. (A) A photograph of a dorsal view of two dorsal neuroepithelial cells filled with Lucifer yellow and treated with an antibody to Lucifer yellow. The dorsolateral endfeet processes lie longitudinally along the lateral border of the cord (denoted by the filled triangles). The cell bodies, which are out of the focal plane, are close to the midline (denoted by the open triangles). (B) A camera lucida drawing of a side view of the spinal cord (S) showing dye-filled dorsal (upper) and ventral (lower) neuroepithelial cells. The notochord (N) is ventral to the cord. Anterior is to the left. Scale (A), 35 μm; (B), 50 μm.

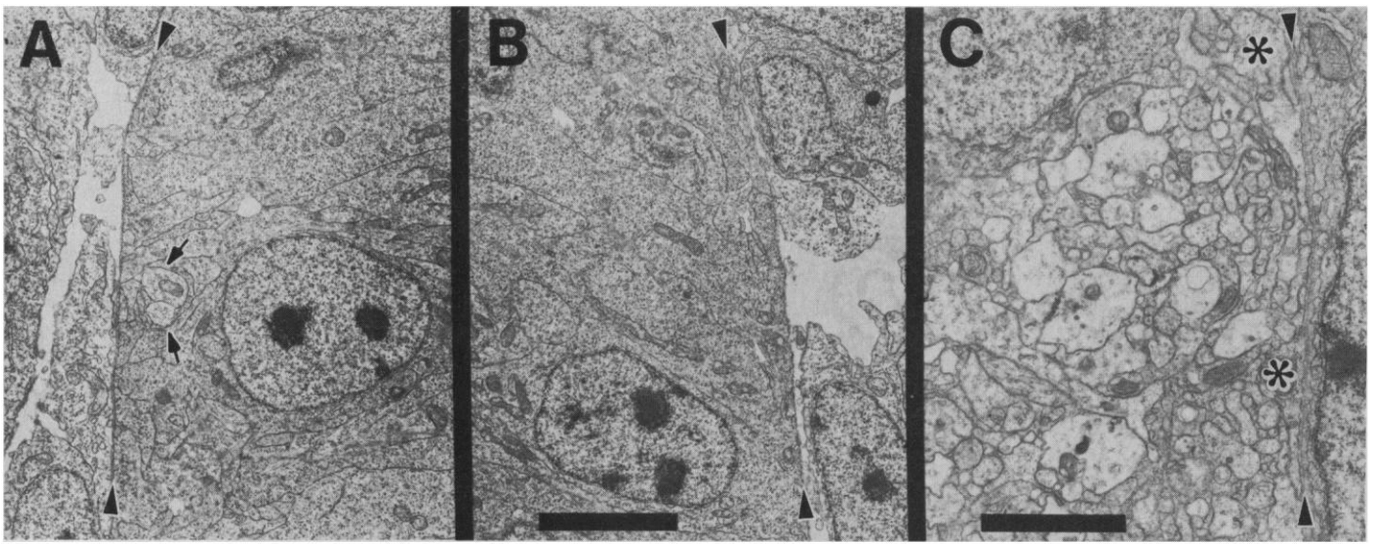

Fig. 8. The morphology of the cord after laser ablation of RB neurons in 40-hour embryos. Axons were distinguished from endfeet processes by their regular arrays of microtubules and light cytoplasm while endfeet processes were darker with lots of ribosomes. (A) An electron micrograph of the dorsolateral region of the cord on the control side 3 hours after the laser ablations. Arrows point to axons in the DLF (presumably RB axons), and the profiles adjacent to the basal lamina are the endfeet processes of dorsal neuroepithelial cells. The basal lamina is denoted by the arrowheads. (B) An electron micrograph of the dorsolateral region of the cord on the experimental side from the same section shown in (A). No debris from laser-ablated RB neurons is evident. Likewise there are no axons on the experimental side, and the dorsolateral endfeet processes appear similar to those on the control side. (C) An electron micrograph of the dorsolateral portion of the cord on the experimental side 60 hours after the laser ablations. Many axon profiles can be seen, and two of the endfeet processes are marked by asterisks. Scale, (A) and (B), 5 μm; (C), 2 μm.

mental segments had anterior axons of normal length (n = 6, Fig. 5A). The DL1 growth cone presumably extended along the RB axons in these segments. These results further confirm the specificity of the laser ablations since both the DL1 neurons, as well as the neuroepithelial cells, appear undamaged by the RB ablations. They also rule out any critical role for neighboring RB cell bodies in the guidance of the DL1 growth cone.

To test the role of RB axons in the guidance of the DL1 growth cone, RB neurons were ablated from one side of a number of segments (four to seven) as described above. In addition, the dorsolateral regions of these experimental segments were mechanically isolated from the normal, flanking segments by scar tissue formed after laser ablation of cells at the segment borders in 40-hour embryos. In this way, the experimental segments were generally free of both RB cell bodies and of axons from RB neurons in the flanking segments. Sixty hours after the laser ablations, the DL1 neurons was assayed by intracellular dye injections. In such embryos, DL1 neurons from control (unmanipulated) hemisegments both ipsilateral and contralateral to the RB-ablated hemisegments were normal (n = 14) (Fig. 9B); such control DL1 neurons had axons that extended anteriorly for four to eight segments.

In contrast, in 14 embryos the DL1 neurons from experimental segments had axons that extended anteriorly for one segment or less (Fig. 1C, 9C) in 14 out of 19 cases, and 1.5 to 2 segments in 2 of 19 cases. Although it is not known whether these growth cones are initially extended and then stop or extend very slowly, these results do suggest that the DL1 growth cones require the RB axons for normal pathfinding. Of the other three cases, the experimental DL1 axons were three, five, and five segments long. The three longest DL1 axons probably extended on RB neurons from the experimental segments which were inadvertently spared (see below).

In general, the laser ablations appeared both effective and specific. First, in most embryos assayed the axons of the RB neurons from control segments flanking the experimental segments were not able to cross over into the experimental segments. In eight of ten cases from six embryos in which both flanking posterior RB neurons and experimental DL1 neurons were assayed, the DL1 axon was short (less than or equal to 1.5 segments long) and the RB axons did not grow into the experimental segments (Fig. 5B). Second, only a few of the approximately 15 to 20 RB neurons from the experimental side of the 4 to 7 segments were not totally eliminated by the laser (Figs. 5B and 9A). There were 0.57 RB neurons (range: 0 to 1.2) per segment on the experimental side and 2.70 RB neurons (range: 2 to 3.8) per segment on the contralateral control side in 14 embryos. In ten of these embryos both spared RB neurons and experimental DL1 neurons were assayed. Of these, seven embryos had ten DL1 neurons with abnormally short axons, and 9 of the 11 spared RB neurons assayed had very stunted axons or no axons at all (*15*). It is likely that the abnormal RB neurons were neurons which were damaged but not killed by the laser. In contrast, the three longest experimental DL1 axons were found in three embryos in which four of six spared RB neurons assayed had relatively normal axons. Third, in six cases from three embryos the experimental DL1, flanking posterior RB neurons, and spared RB neurons were assayed. In all six cases the DL1 axon was short, the flanking posterior RB axons did not cross into the experimental segments, and the spared RB neurons had only very stunted axons or no axons.

The stunting of the DL1 axons in the experimental segments appeared not to result simply from damage to the DL1 neurons (*16*) or from nonspecific effects on growth cone extension. This is indicated by four observations. First, in the control experiments described above, when experimental segments were not isolated, RB axons from flanking segments extended through the ablated segments, and DL1 axons extended anteriorly along them. Second, electron micrographs of experimental embryos indicated that the endfeet processes of the dorsal neuroepithelial cells were normal in the experimental segments (see earlier section). Third, axons of the adjacent DM1 (n = 4) and C1 (n = 5) neurons in these experimental segments extended normally (Fig. 5C). Fourth, the contralateral C1 neuron extended its axon across the midline and then into the tract just ventral to the DLF in experimental segments (n = 3). These results indicate that laser ablations of RB neurons specifically

kill the RB neurons, and as a result specifically alter the guidance of the DL1 growth cones.

Neuronal pathfinding by growth cones in the invertebrate and vertebrate CNS. The growth cones of identified neurons in the developing spinal cord of the fish embryo extend along stereotyped and precise pathways from the outset, just as do other neurons in both vertebrate (chick and fish) and invertebrate (insect and leech) embryos (*17*). Furthermore, the experimental results presented here suggest that the growth cones of these spinal neurons are guided by several types of cellular cues, including specific nonneuronal and neuronal surfaces. The data are consistent with the hypothesis that the RB growth cones, which pioneer the DLF, recognize cues on specific subsets of neuroepithelial cells. The DL1 neurons subsequently fasciculate with the RB axons in the DLF and in doing so appear to specifically recognize cues on the RB axons.

During normal development, the growth cones from RB neurons use as their substrates both other RB neurons, and the endfeet of dorsal neuroepithelial cells. When RB neurons are laser ablated in a number of experimental segments, RB axons from flanking segments enter and traverse the experimental segments by extending along their normal pathway. Degenerating remnants of laser-ablated RB neurons are gone within 3 hours of the ablations and probably do not provide guidance cues for the flanking RB growth cones (*18*). These results suggest that neuronal surfaces are not necessary, but rather that the endfeet of dorsal neuroepithelial cells may provide the guidance cues for RB growth cones. In addition, since RB filopodia have access to more ventral neuroepithelial endfeet but RB growth cones do not extend along them, the guidance cues for the RB growth cones may be differentially restricted to the endfeet of the dorsal neuroepithelial cells.

It seems quite likely that the growth cones which pioneer the more ventral longitudinal tracts may also exhibit a specific attraction for the endfeet of more ventral neuroepithelial cells. Thus, longitudinal arrays of neuroepithelial processes might provide preformed pathways for their respective pioneering growth cones. In this way the basic set of longitudinal tracts in the developing spinal cord could be established by a simple orthogonal pattern of nonneuronal cells. These results are thus consistent with the "blueprint hypothesis" proposed by Singer and his colleagues for the developing vertebrate spinal cord (*19*), and further generalized for preformed neuroepithelial and glial pathways for other regions of the CNS (*20*).

However, in addition to nonneuronal cells, neuronal surfaces also play an important role in the guidance of neuronal growth cones. The DL1 neurons appear to fasciculate with the RB axons in the DLF. The laser ablation experiments demonstrate the importance of the RB axons in the guidance of the DL1 growth cones. The growth cones of many spinal neurons extend along already established tracts, and it seems quite likely that many of these may use specific guidance cues on the axons within these tracts. These results thus support the "labeled pathways hypothesis" proposed by Goodman and his colleagues for the developing grasshopper CNS (*21*). In both organisms, follower growth cones appear to specifically recognize particular bundles of axons, suggesting that these axons are differentially labeled. Such axonal cues are likely to be highly specific in the fish spinal cord since in the absence of RB neurons, DL1 growth cones seem to ignore the axonal substrates provided by other nearby cells, both neuronal and nonneuronal, that should be within filopodia reach: the adjacent C1 and DM1 neurons and their substrates, and the endfeet processes of dorsal neuroepithelial cells in the region of the DLF.

Although my results show the importance of specific neuronal recognition for growth cone guidance, they do not indicate the exact nature of these specific interactions. For example, the RB axons might provide the DL1 growth cones with specific growth-promoting information, or with specific pathway information, or both. Since, in the absence of the RB neurons, the stunted DL1 axons are still oriented in the correct direction, it is possible that interactions with RB neurons may be necessary only to specifically promote DL1 growth rather than to provide pathway information. It is difficult to distinguish between these two alternatives.

However, other spinal growth cones, such as those of C1 neurons, do change their pathway at a specific choice point while switching from their circumferential pathway to their longitudinal pathway. Since the C1 neuron has already initiated growth before reaching this choice point, it is likely that growth-promoting interactions are not solely involved here. Rather, it is likely that specific cellular cues guide the C1 growth cone to turn along its specific longitudinal tract. Similarly, in the developing CNS of the grasshopper embryo, the G growth cone extends across the midline along a commissural axonal pathway, and then turns anteriorly along a particular longitudinal axonal pathway by specific recognition of the axons in this particular tract (*5*).

Neuronal specificity is generally a product of pathfinding by growth cones followed by synapse formation and stabilization. Recognition of specific cellular cues has now been implicated for growth cone pathfinding in a number of systems both vertebrate and invertebrate. Further detailed analysis of the developing spinal cord of the fish embryo may reveal how neuronal specificity is achieved in the vertebrate nervous system.

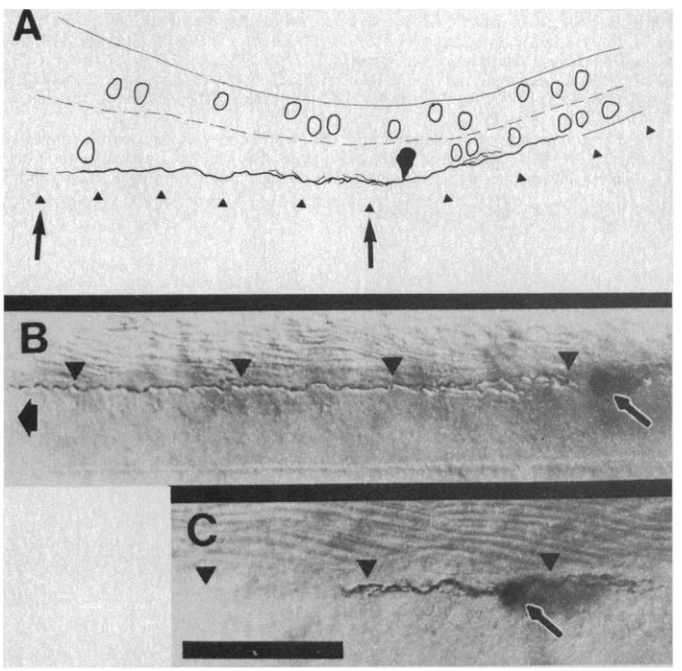

Fig. 9. Pathfinding by RB and DL1 neurons after laser ablations. (A) Camera lucida drawing of a dorsal view of an RB neuron from the segment posterior to the segments (between the arrow) from a 100-hour embryo in which the RB neurons had been laser-ablated 60 hours before. All the other RB neurons in the segments shown are outlined. Dashed line marks the midline of the cord, and the triangles mark the segment borders. (B) Photograph of a dye-filled DL1 from a control segment in a 100-hour embryo in which the RB neurons were laser ablated from one side of four segments and the dorsal lateral portions of those segments were isolated from the adjacent control segments at 40 hours of development. Arrow designates that the DL1 axon, which was eight segments long, extends beyond the limits of this photograph. Small arrow points to the DL1 cell body. (C) Photograph of a dye-filled DL1 from the experimental side of a segment from a 100-hour embryo that received the same manipulations as described in (B). This neuron was from segment 6; the experimental segments were 4 through 8. Scale (A), 150 μm; (B) and (C), 50 μm.

REFERENCES AND NOTES

1. Ramón y Cajal, *Anat. Anz.* **5**, 609 (1890); *Studies on Vertebrate Neurogenesis*, L. Guth, transl. (Thomas, Springfield, IL, 1929); R. G. Harrison, *J. Exp. Zool.* **9**, 787 (1910).
2. M. Singer, R. H. Nordlander, M. Egar, *J. Comp. Neurol.* **185**, 1 (1979).
3. J. Silver, D. Wahlsten, J. Coughlin, *ibid.* **210**, 10 (1982); J. Silver and M. Y. Ogawa, *Science* **220**, 1067 (1983); J. Silver and U. Rutishauser, *Dev. Biol.* **106**, 485 (1984).
4. C. Lance-Jones and L. Landmesser, *J. Physiol. (London)* **302**, 559, 581 (1980); V. Whitelaw and M. Hollyday, *J. Neurosci.* **6**, 1199, 1216, 1226 (1983).
5. J. A. Raper, M. Bastiani, C. S. Goodman, *J. Neurosci.* **3**, 20, 31 (1983); *ibid.* **9**, 2329 (1984); M. Bastiani, J. A. Raper, C. S. Goodman, *ibid.*, p. 2311; C. S. Goodman *et al.*, *Science* **225**, 1271 (1984).
6. M. Bastiani, C. Q. Doe, S. L. Helfand, C. S. Goodman, *Trends Neurosci.* **8**, 257 (1985).
7. C. B. Kimmel, S. L. Powell, W. K. Metcalfe, *J. Comp. Neurol.* **205**, 112 (1982); C. B. Kimmel, W. K. Metcalfe, E. Schabtach, *ibid.* **233**, 365 (1985); P. Z. Myers, *ibid.* **236**, 555 (1985).
8. Medaka embryos were collected daily from fish maintained in a breeding colony, and kept in petri dishes at 21°C; stage was designated according to criteria described in R. V. Kirchen and W. R. West, *The Japanese Medaka* (Carolina Biological Supply, Burlington, NC, 1976).
9. In order to analyze the morphology of the identifiable neurons in the embryonic fish spinal cord, which consists of 30 segments, the chorion and yolk sac were removed with forceps and scissors, the skin covering the spinal cord was dissected off, and the embryo was pinned out dorsal side up in a sylgard-lined chamber filled with Yamamoto's saline [T. Yamamoto, *Int. Rev. Cytol.* **12**, 361 (1961)]. In some cases the skin was left on so that peripheral axons could be examined. The embryo was fixed by immersion in 2 percent paraformaldehyde and 1 percent dimethyl sulfoxide (DMSO) in 100 m*M* sodium phosphate buffer (*p*H 7.4) for 10 to 15 minutes and then washed several times with buffer. The chamber was placed on a fixed-stage compound microscope, neurons from spinal cord segments 3 to 20 were visualized with Nomarski optics, and each was impaled and dye-filled with microelectrodes containing a 10 percent solution of the fluorescent dye Lucifer yellow. Embryos were then further fixed for 1/2 to 1 hour and washed several times; the dye-filled neurons were visualized with Nomarski optics with the use of a rabbit antiserum to Lucifer yellow [P. H. Taghert, M. J. Bastiani, R. H. Ho, C. S. Goodman, *Dev. Biol.* **94**, 391 (1982)].
10. J. S. Eisen, P. Z. Myers, M. Westerfield, *Nature (London)* **320**, 269 (1986).
11. J. D. W. Clarke, B. P. Hayes, S. P. Hunt, A. Roberts, *J. Physiol. (London)* **348**, 511 (1984); N. C. Spitzer, *Trends Neurosci.* **7**, 224 (1984).
12. J. D. W. Clarke *et al.*, in (*11*); A. Roberts and B. P. Hayes, *Proc. R. Soc. London Ser. B* **196**, 415 (1977); A. Roberts and J. D. W. Clarke, *Philos. Trans. R. Soc. London Ser. B* **296**, 195 (1982).
13. For examination of the ultrastructure, the identifiable neurons were microinjected with a 4 percent solution of horseradish peroxidase and fixed with a solution of 3 percent glutaraldehyde, 2 percent paraformaldehyde, 1 percent acrolein, and 1 percent DMSO in phosphate buffer (100 m*M*, *p*H 7.4) for 1 hour. The embryos were then postfixed in 2 percent osmium tetroxide in phosphate buffer for 2 hours, stained with 2 percent uranyl acetate in water for 1 to 2 hours, dehydrated in an ethanol and propylene oxide series, embedded in plastic, and thin-sectioned.
14. The RB neurons were ablated from one side of three to seven segments in 40-hour embryos by visualizing the neurons in ovo with a compound microscope through which a pulsed, dye laser microbeam was focused. Immediately after exposure to the laser microbeam the nuclei of the neurons became bloated, and their cytoplasm became granular. After several minutes the neurons had shriveled considerably or had broken up into small fragments. In one set of these embryos the dorsolateral portions of the experimental side of the RB-ablated segments were isolated from the adjacent unmanipulated segments by repeated shots with the laser microbeam. Before treatment with the laser, the eggs were placed in water with CO_2 bubbling through it for 5 to 10 minutes. This temporarily immobilized the medaka embryos, which normally have yolk sacs that spontaneously contract [R. Fluck, R. Gunning, J. Pelligrino, T. Barron, D. Panitch, *J. Exp. Zool.* **226**, 245 (1983)]. The contractions returned within 10 minutes of this treatment by bubbling air into the water following the laser ablations. This procedure did not appear to affect the development of the identifiable neurons in the spinal cord.
15. In three embryos the experimental segments were completely devoid of RB neurons or all the spared RB neurons were assayed and found to be abnormal. In these embryos the four experimental DL1 neurons assayed all had short axons.
16. In a small number of cases the experimental DL1 may have been inadvertently damaged by the laser during ablation of the RB neurons. This is indicated by the two of ten cases in which the experimental DL1 axon was short but the posterior RB axons did cross through the experimental segments. Likewise, in two of ten cases the experimental DL1 was short, but a spared RB neuron had relatively normal axons.
17. C. Lance-Jones and L. Landmesser, *Proc. R. Soc. London Ser. B.* **264**, 1 (1981); J. S. Eisen, P. Z. Myers, M. Westerfield, *Nature (London)* **320**, 269 (1986); C. S. Goodman and M. Bastiani, *Sci. Am.* **251**, 58 (December 1984); J. Y. Kuwada and A. P. Kramer, *J. Neurosci.* **3**, 2098 (1983).
18. At 43 hours of development, degenerating remnants of RB neurons that were subjected to laser ablation at 40 hours were no longer evident. In this interval, RB growth cones normally extend a single segment from their cell bodies. Therefore, all experimental segments, except for the most anterior segment and the most posterior segment, should be free of degenerating RB remnants. Thus, the growth cones of RB neurons from flanking segments can grow through up to five segments devoid of RB neurons and their remnants.
19. M. Singer, R. H. Nordlander, M. Egar, *J. Comp. Neurol.* **185**, 1 (1979).
20. J. Silver and U. Rutishauser, *Dev. Biol.* **106**, 485 (1984).
21. J. A. Raper, M. Bastiani, C. S. Goodman, *J. Neurosci.* **3**, 31 (1983).

22. I thank M. J. Bastiani for help with some of the electron microscopy, C. S. Goodman for use of facilities, E. Ockerman for the computer-generated artwork, and M. J. Bastiani, C. S. Goodman, and M. S. Horton for critical reading of the manuscript. Supported by NIH grant NS 20299 to J.Y.K. and NIH grant HD 21294 to C.S.G.

18 March 1986; accepted 30 June 1986