

The Role of Microtubules in Growth Cone Turning at Substrate Boundaries

Elly Tanaka and Marc W. Kirschner

Department of Biochemistry, University of California, San Francisco, California 94143-0448

Abstract. To understand the role of microtubules in growth cone turning, we observed fluorescently labeled microtubules in neurons as they encountered a substrate boundary. Neurons growing on a laminin-rich substrate avoided growing onto collagen type IV. Turning growth cones assumed heterogeneous morphologies and behaviors that depended primarily in their extent of adhesion to the substrate. We grouped these behaviors into three categories—sidestepping, motility, and growth-mediated reorientation. In sidestepping and motility-mediated reorientation, the growth cone and parts of the axon were not well attached to the substrate so the acquisition of an adherent lamella caused the entire growth cone to move away from the border and consequently reoriented

the axon. In these cases, since the motility of the growth cone dominates its reorientation, the microtubules were passive, and reorientation occurred without significant axon growth. In growth-mediated reorientation, the growth cone and axon were attached to the substrate. In this case, microtubules reoriented within the growth cone to stabilize a lamella. Bundling of the reoriented microtubules was followed by growth cone collapse to form new axon, and further, polarized lamellipodial extension. These observations indicate that when the growth cone remains adherent to the substrate during turning, the reorientation and bundling of microtubules is an important, early step in growth cone turning.

NUMEROUS experiments, particularly cell transplantation experiments suggest that the intricate paths that neurons take to their targets are determined by cues in the environment, rather than an intrinsic program within each cell (9, 11, 18). Therefore some mechanism must exist to transduce the extracellular signals into the cell so that it can elaborate the proper cellular structure. Morphologically, the growth cone seems to be the site where these signals are integrated to guide axon formation in the proper orientation (12, 21). The growth cone is a highly dynamic structure, continuously extending and retracting long, actin-rich spikes called filopodia and thin, flat veils called lamella in apparently highly variable and perhaps random directions (3). Though it is very likely that the actin-rich extensions of the growth cone are the sites of adhesion and motility, it is much less clear how the growth cone selects which direction to extend and grow.

Growth cones both in culture and in vivo adopt variable morphologies during turning. Although ultimately an asymmetry in the shape of the growth cone is established, the subcellular basis for this asymmetry is unknown. In some situations, neurons transiently form two distinct branches with only one of the two branches eventually surviving, the other

being retracted (4, 25). In other cases, growth cones slowly reorient their movement, or extend more filopodia in the direction of eventual growth (16, 25). Time lapse studies of growth cones during turning showed that new axon forms when organelle-rich cytoplasm invades the lamella. Subsequently, the other dynamic regions of the growth cone collapse around the organelle-rich region to form the new axon (1, 8). It is likely that microtubules are involved at some early step in choosing the lamella that will eventually be transformed into axon. First, microtubules are the tracks along which neuronal organelles move so the invasion of lamella by organelles most likely reflects a previous distribution of microtubules (5, 20, 22). Second, observation of fluorescently tagged microtubules during growth cone guidance showed that microtubules extended deep into branches or lamella that would eventually become the new axon (19).

Because most previous studies used fixed samples or low time resolution observations to study the turning process in neurons, it is unclear what sequence of microtubule rearrangements occur in the initial choice of a lamella for invasion and the subsequent process by which the entire microtubule array is recruited to form a coherent axon bundle. To address these questions, we have chosen to follow microtubules in the growth cones of cultured *Xenopus* neurons as they make turning decisions. We have used as a model the encounter at a border between favorable and unfavorable substrates. Using the system of explanted neurons from the neural tube of *Xenopus* embryos, which had been previously labeled with fluorescently tagged tubulin, we could follow

Address correspondence to Dr. E. Tanaka, Ludwig Institute for Cancer Research, Middlesex Hospital/University College Branch, 91 Riding House Street, London, W1P 8BT, England.

The present address for Dr. Marc W. Kirschner is Department of Cell Biology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115. Tel.: (617) 432-2200. Fax: (617) 432-0420.

microtubules with high spatial and temporal resolution. In this paper, we describe the sequence of microtubule rearrangements that occur during turning decisions at a substrate border.

From these experiments, we find that growth cone guidance appears to be a multi-step process where a lamella must first form a stable attachment to the substrate so that microtubule bundles can ultimately invade that structure. When multiple stable lamella are formed, single microtubules are seen to extend first into the periphery of one lamella, followed by the recruitment of the entire microtubule mass in the growth cone into that lamella, followed by bundling of that mass, with subsequent extrusion of the bundle to form the new axon.

Materials and Methods

Preparation of Patterned Substrate

(A) Collagen coating: 25-mm diam round coverslips were washed with 1 N HCl for 14 h at 65°C, and then washed extensively with glass distilled H₂O and air dried. Before the experiment, coverslips were coated with 150 μ l of 200 μ g/ml collagen type IV (Collaborative Research, Bedford, MA). After incubation for 1–3 h, coverslips were rinsed with distilled H₂O and air dried. (B) Producing Matrigel stripes. Concentrated Matrigel (Collaborative Research) was stored in small (10 μ l) aliquots at –20°C. To make stripes, stock Matrigel was diluted 50-fold, and then labeled with 2×10^{-6} M *N*-hydroxy-succinimide-ester-coumarin (kind gift of Tim Mitchison). The mix was then incubated at 37°C for 1 h in the dark to gel the Matrigel. Stripes of Matrigel were then aspirated into 0.1- μ m nucleopore polycarbonate filters (Costar Corp., Cambridge, MA) as described for membrane particles (27). Matrigel-patterned filters were stored on top of a thin layer of 1% agarose/Ca⁺⁺, Mg⁺⁺-free PBS at 4°C. To make alternating stripes of Matrigel and collagen, the filter plus the agarose underneath it were excised from the rest of the surrounding agarose by cutting a square around the filter with a razor blade. This block was carefully picked up with forceps and inverted onto the dried, collagen-coated coverslip so that the Matrigel was facing the collagen-coated surface. This assembly was incubated for 30–60 min at room temperature. The coverslip was then flooded with Ca⁺⁺, Mg⁺⁺-free PBS, and the agarose block, and the filter was removed. All coverslips were checked for proper patterning (*sharp stripes*) with epifluorescence microscopy before use. Just before plating explants, the PBS was replaced with Steinberg's plating media.

Neural Cultures and Microtubule Labeling

Labeling of neuronal microtubules and culturing of neural tube explants was performed as described previously (26). Briefly, *Xenopus* eggs were fertilized in vitro as described previously. At the two-cell stage the eggs were injected with 50 nl rhodamine-labeled tubulin. After the eggs developed to stage 22–24, the dorsal portion of the embryo was excised and incubated in 1 mg/ml collagenase A in Steinberg's solution. After 40 min, neural tubes were dissected from the dorsal tissue and explanted onto patterned coverslips (described above) in Steinberg's media with 20% L-15 media. After 8–12 h, turning neurons were observed. Imaging of cultured neurons was also performed as previously described (26). Briefly, cultures were placed in a sealed, deoxygenated chamber. Cells were observed using an Olympus IMT-2 microscope, 60X/1.3 NA objective. Images were collected using a Photometrics cooled CCD camera (Tucson, AZ) with a Texas Instruments chip at 0.1-s exposures with 75% attenuated mercury light. Images were stored and processed using a micro VAX computer using the software and integrated hardware described previously.

Results

Xenopus Neurons Turn at a Substrate Border

To produce turning decisions of growth cones, we chose patterned substrates of different extracellular matrix molecules

(ECMs).¹ It seemed likely that the neurons emerging from cultured *Xenopus* neural tube explants would respond to borders of different ECM molecules, even if these preparations of ECM did not reproduce exactly the components found in the embryo. Laminin or laminin-rich crude substrates such as Matrigel induced rapid outgrowth while in contrast, explants cultured on fibronectin or collagen type IV very poorly supported outgrowth (data not shown). We tested whether neurons growing on Matrigel might turn to avoid collagen as had previously been shown for dorsal root ganglia from chick (10). Patterns of ECM were prepared by adapting a method used for guidance assays on membranes from different regions of chick optic tectum (see Materials and Methods). The substrate pattern was visible using epifluorescence (Fig. 1 *a*). 8–15 h after plating on the patterned coverslips, the neural tube explants had extended neurites over Matrigel-containing regions but had avoided collagen, resulting in patterns of neurite outgrowth that matched the patterns of fluorescent stripes (Fig. 1 *b*).

1. Abbreviation used in this paper: ECM, extracellular matrix molecule.

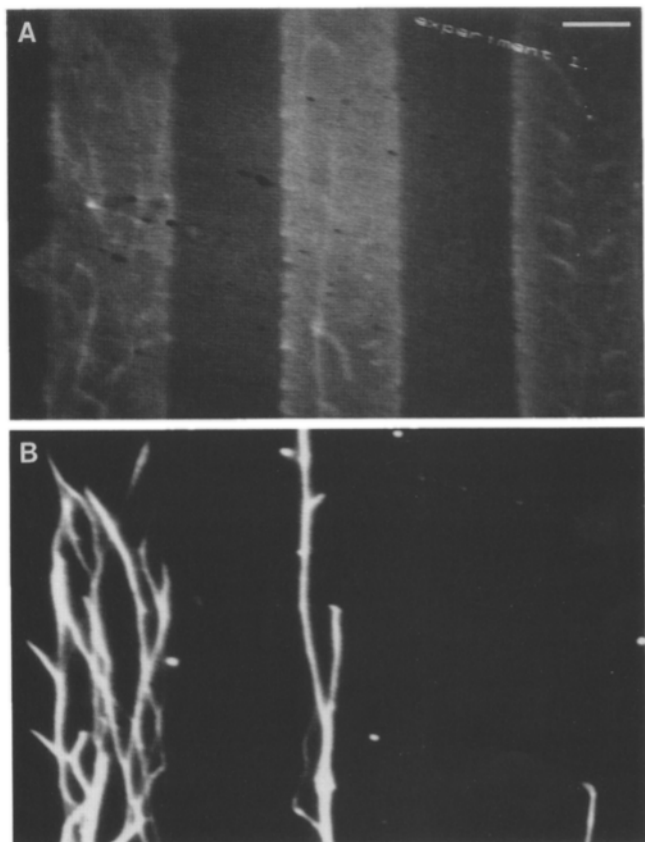


Figure 1. *Xenopus* neurons follow patterns of Matrigel and avoid collagen. (A) Alternating 100- μ m stripes of Matrigel (white stripes) and collagen (dark stripes) were made as described in Materials and Methods. The Matrigel was fluorescently labeled with coumarin. (B) Matched picture of neurons grown on the patterned substrate. Neurons follow the stripes of Matrigel. In this picture, neurons were fixed and visualized by immunofluorescence against tubulin. Each fine strand represents an axon or multiple, fasciculated axons. Bar, 55 μ m.

Observation of Growth Cones during Turning at Substrate Borders

To observe microtubules during turning decisions, neural tube explants from rhodamine-tubulin-injected *Xenopus* eggs were plated onto the patterned coverslips, and growth cones approaching substrate borders were observed 8–12 h after plating. Both the growth cone and the microtubules could be followed simultaneously using rhodamine epifluorescent illumination, since the general shape of the growth cone was visible from the background signal of the rhodamine-labeled tubulin monomer. The turns (measured from the first indication of a change in axonal direction to a point of clear reorientation) took between 10 and 87 min averaging 28 min. In total we observed 33 neurons growing on Matrigel that encountered a collagen border. In seven of the sequences the growth cone interacted with other cells during the turn obscuring and complicating its behavior while in three cases the growth cone had already initiated turning before observation. These observations were not included in this study. Of the 23 remaining sequences, two growth cones remained paused at the border throughout the observation period and two growth cones grew through the border without turning (one growth cone reoriented at the border, and then grew through the border, so it was counted in two categories), four suddenly reacted after reaching the border, and two of these grew back in a new direction, successfully avoiding the border; the other two failed to grow back during the observation period. The remaining 16 neurons changed their direction of movement at the substrate border. It is these events that we shall describe below.

The average angle at which the 23 neurons approached the border was 52 ± 23 degrees. There was no significant differ-

ence in the average approach angle for the neurons that displayed collapsing, sidestepping, or turning behaviors at the border (51 ± 45 , 52 ± 25 , and 46 ± 18 , respectively, see below). In the two instances in which the growth cone grew through the border, the approach was nearly perpendicular at 84° and 90° .

Three Distinct Classes of Turning Behavior at Substrate Borders

We examined 16 growth cones that clearly changed their direction of movement at the border. We could categorize their behavior into three distinct classes: sidestepping, motility-mediated reorientation, and growth-mediated reorientation. The primary criteria we used to categorize the neurons was whether the reorientation of the microtubule bundle occurred behind the growth cone by the bending of the axon, or whether the microtubules were redirected within the growth cone during turning. This is schematized in Fig. 2. We do not mean to imply that these classes reflect different mechanisms of turning, but merely clearly defined morphological categories of behavior. Some of the neurons exhibited two behaviors consecutively during the encounter with the border and were counted twice.

Sidestepping. Four neurons underwent what we term “sidestepping” behavior, in which the growth cone moved laterally along the border without a change in the local orientation of the axon, as diagrammed in Fig. 2. In these neurons both the central part of the growth cone and the axon did not remain stably attached to the substrate so as the growth cone lamella spread and attached to the Matrigel, the entire growth cone and axon moved toward the lamella laterally along the border (Fig. 2 *B* and *C*). During this process, the

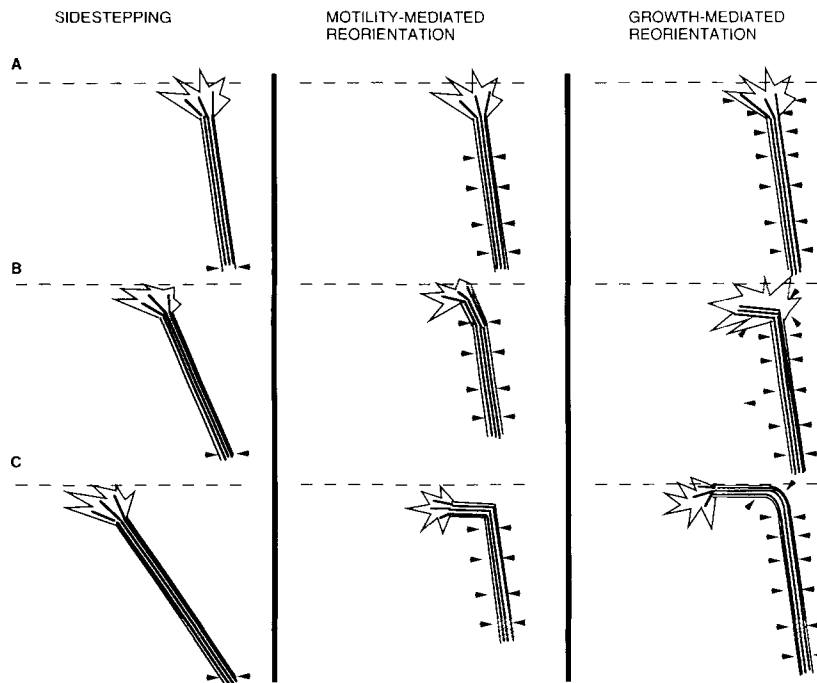


Figure 2. Three types of turning—sidestepping, motility- and growth-mediated reorientation—differ in the degree of adhesion to the substrate. (*Sidestepping*) (*A*) The growth cone at the border is not well adhered to the substrate, and the axon is adherent at a point distant from the growth cone neck. (Arrowheads mark the areas that adhere strongly to the substrate). (*B*) As the growth cone moves laterally along the border to avoid the collagen, the non-adherent axon also moves laterally without forming a bend. (*C*) The growth cone moves further laterally, and the axon is growing as its angle to the border becomes steeper. (*Motility-mediated reorientation*) (*A*) The growth cone approaching the border is not well adherent to the substrate, but the axon is adherent until close to the growth cone neck (arrowheads). (*B*) The lateral lamella makes stable contact with the substrate and the entire growth cone begins to move in that direction. Since the axon behind the growth cone neck is not adherent, it gets turned laterally. (*C*) As the entire growth cone starts moving along the border, the axon behind it has become reoriented, bending at the point of adhesion. The microtubules follow the movements of the growth cone. (*Growth-mediated reorientation*) (*A*) The growth cone and the axon are adherent along their lengths. (*B*) As the growth cone maintains contact with the substrate, the microtubules within change their orientation and begin to form bundles. (*C*) The growth cone has collapsed around the reoriented microtubule bundle, and new lamellipodial extension has initiated at the end of the reoriented axon.

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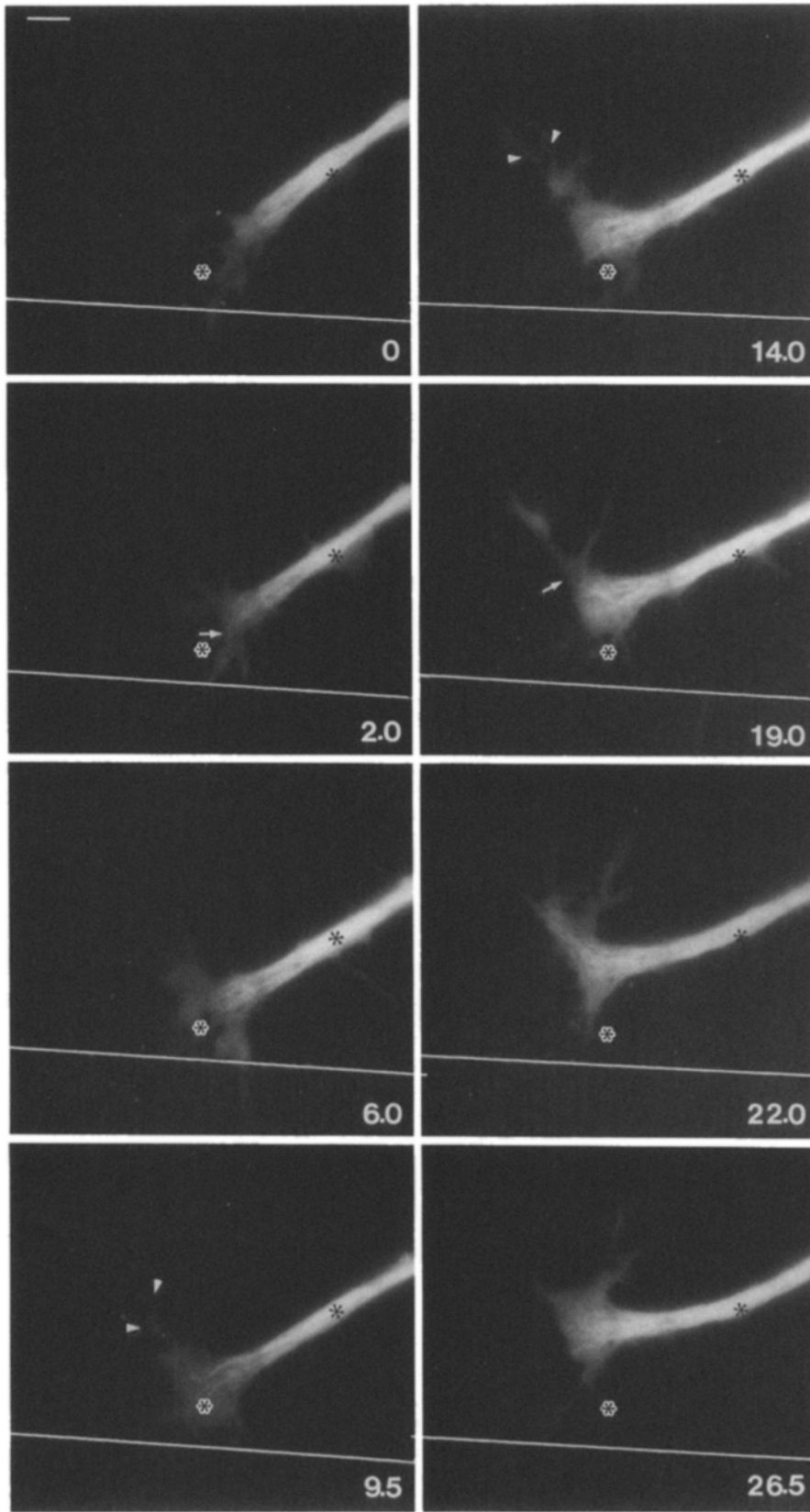


Figure 3. Turning at a substrate border by motility-driven reorientation. (A) Fluorescent images of a turning neuron. (B) Matching traces of cell outline and microtubules in the growth cone. In this sequence the line denotes the border between Matrigel (above) and collagen (below). (0 min). The growth cone reached the border and extended a lamella across onto the collagen (c lamella in B). Microtubules remained well behind the border (2 min). The c lamella shrank and the upper lamella on Matrigel (m lamella) expanded. A single microtubule bent into the base of the c lamella (arrow). (6.0 min). The m and c lamellae are similar in size while microtubules remain at their bases. (9.5 min) The m lamella (in B) expanded laterally and very little of the c lamella extends across on collagen. (14 min) Lateral m lamella remained stable (arrowheads) and microtubules began to fill the growth cone. The c lamella shrank. (19 min) The m lamella continued to extend laterally, and a few microtubules have bent to enter the base of the m lamella (arrow). The c lamella collapsed close to the microtubule bundle. (22.0 min) The lateral m lamella has expanded, and two microtubules entered deep into it (near asterisk). The remnants of the c lamella continued to lose adherence. The movement of the growth cone away from the border created a gentle bend in the axon behind the growth cone neck. (26.5 min) The c lamella has lost most of its attachments close to the border. Consequently, the whole growth cone moved away from the border. This can be seen both by the increased distance between the growth cone and the border. The asterisks mark constant positions in the field. The growth cone translocates away from the lower left asterisk dragging the distal portions of the axon with it while the upper right asterisk marks a relatively stationary portion of the axon. Bar, 5 μ m.

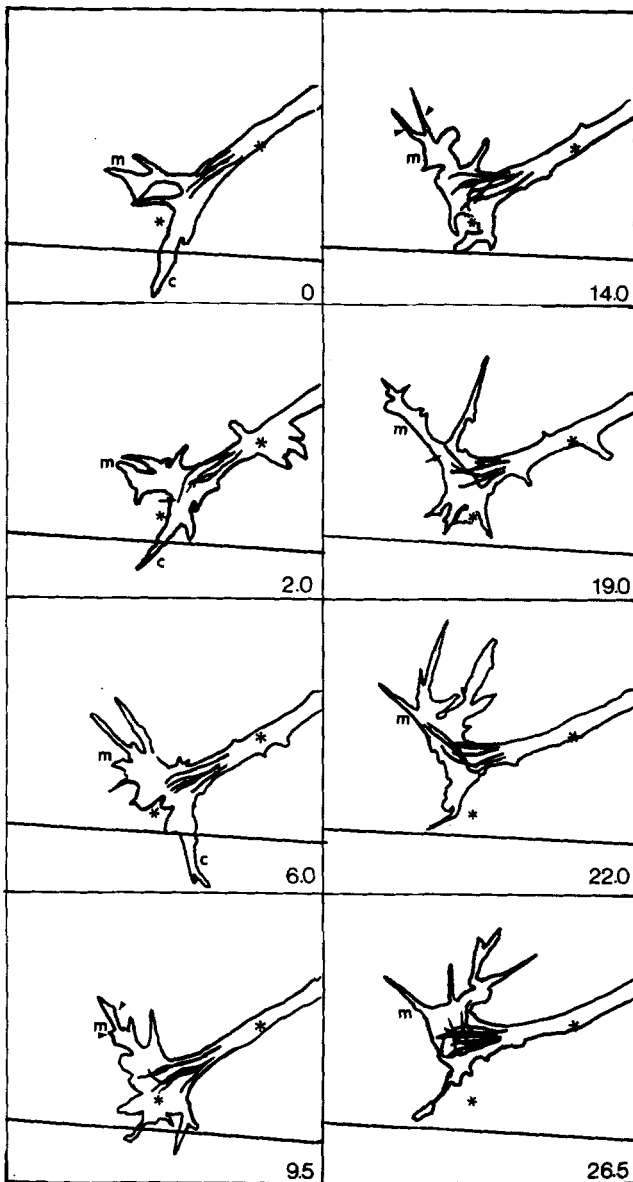
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Figure 3 (continued).

axon remained straight as the growth cone crawled laterally (data not shown). The microtubule behavior was unperturbed by these movements and resembled that found in neurons extending straight on uniformly coated coverslips, as described previously (26). The movement resembled that of a dog on a leash, where the dog is the growth cone and the leash, the axon.

Motility-mediated Reorientation. In five cases, the growth cone at the border did not maintain strong contacts with the substrate during turning. However, unlike the case of sidestepping neurons, the axons behind the growth cones remained attached to the substratum (*arrowheads*), Fig. 2, A-C). When these growth cones approached borders they extended lamellae or branches in multiple directions (Fig. 2 A), including across the border onto collagen, but most of the lamellae retracted. Eventually, a lamella on Matrigel

formed a stable attachment to the substrate (Fig. 2 B) and the entire growth cone translocated toward the lamella into a new orientation. Since the whole growth cone translocated, the entire microtubule array in the growth cone was forced to move laterally with the growth cone. The configuration of the microtubules in the growth cone did not change dramatically during motility-mediated reorientation, i.e., splayed microtubules did not bundle during turning and reorientation occurred with little net growth of either the microtubule array or the axon. Since the axon behind the growth cone was attached to the substratum, when the growth cone moved away from the border, a bend formed in the axon behind the growth cone to reorient the distal portion of the axon (Fig. 2 C). The behavior of the growth cone resembled that of a fibroblast that was leashed to an adherent axon.

Fig. 3, A and B shows a neuron turning by motility-mediated reorientation. At 0 min, the growth cone approached the border, and extended a lamella (the c lamella, Fig. 3 B) across the collagen border marked by the stripe. By 2.0 min, the upper lamella (the m lamella) on the Matrigel expanded, while the c lamella shrank slightly. Note, however, that some microtubules had invaded the base of the c lamella (*arrow*). At 6.0 min, the growth cone assumed a fairly symmetric shape with microtubules contained in the central region. At 9.5 min the growth cone began expanding the m lamella (*arrowheads*) perpendicular to the axon. At 14.0 and 19.0 min, the m lamella lengthened while the c lamella continued to retract from the border. By 22.0 min, microtubules entered the growing m lamella. However, at 26.5 min, most of the c lamella had detached and the m lamella pulled the growth cone and the distal portion of the axon away from the border. This tugging bent the distal end of the axon parallel to the border. The asterisks mark reference points in the field throughout the turning sequence. At 9.5 min, the asterisk in the lower left coincides with the central portion of the growth cone. As the growth cone turns, it translocates away from the border so that by 26.5 min, the asterisk that marked the position of the growth cone at 9.5 min is no longer located on the growth cone or the axon. In contrast, the upper right asterisk consistently marks the axon, as that portion of the axon has not translocated significantly.

Growth-mediated Reorientation. The other major class of turning was exhibited by nine neurons in our study. In these cases the growth cone maintained contact with the substrate so that turning could not be accomplished by dragging the axon or the growth cone (Fig. 2, *arrowheads*). Instead, turning was accomplished by redirected growth and was accompanied by the reorientation of microtubules within the growth cone (Fig. 2 B), invasion of a lamella by microtubules, and directed elongation of microtubules into that lamella. After elongation of the microtubule bundle, the selected lamella collapsed around the bundle followed by further polarized extension of that lamella (Fig. 2 C).

Two examples of growth-mediated reorientation are shown in Figs. 4 and 5. In Fig. 4 at 0 min, a single microtubule (*arrow*) extended into the peripheral lamella on Matrigel. This microtubule was immediately accompanied by the invasion of other microtubules into that region at 1.5 min (*arrow*) so that by 5.5 min, a substantial bundle of microtubules accumulated and a new lamella (*arrowhead*) formed at the end of the bundle. By this time, a sharp axonal bend formed where the growth cone originally encountered

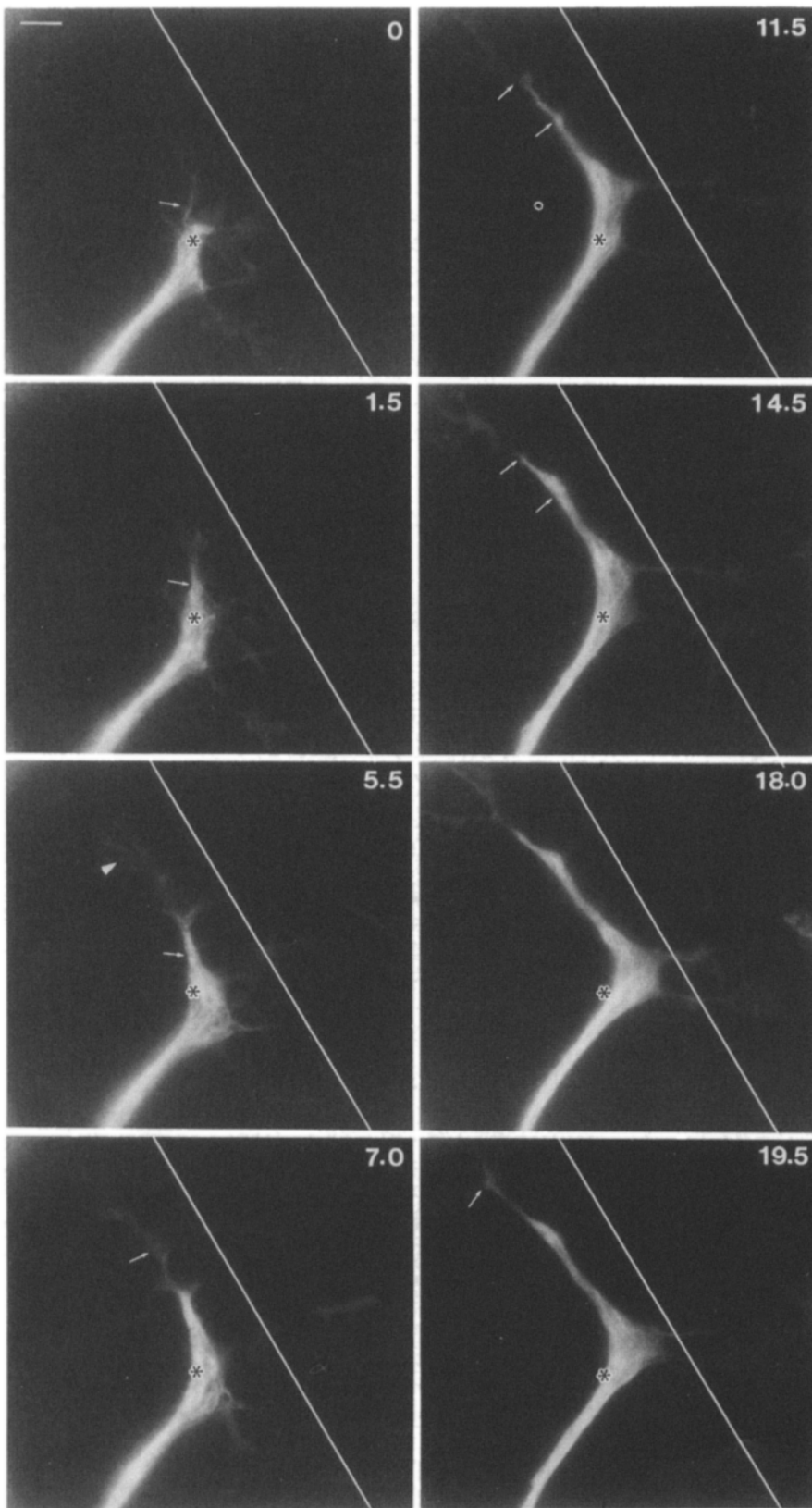
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Figure 4. Turning by growth-mediated re-orientation. (A) Fluorescence images of a turning growth cone and (B) traces of cell outlines and microtubules for the growth cone. (0 min) As the growth cone approached the border, a single microtubule entered the peripheral lamella (arrow). (1.5 min) The region explored by the microtubule at 0 min was invaded by more microtubules (arrow at 0, 1.5, and 5.5 min marks the same point in the field). (5.5 min) Microtubules invading the region consolidated into a thick bundle (arrow) and new lamellipodia sprouted at the end of the bundle (arrowhead). The microtubule array at the border formed a discrete bend. (7.0 min) A single curled microtubule (arrow) extended into the lamella from the distal end of the microtubule bundle. (11.5 min) A few microtubules have followed the single microtubule, so that a thin bundle forms in the region marked by the arrow at 7.0 and 11.5 min. From the end of the bundle emanates a small curled microtubule (upper arrow). (14.5 min) More microtubules invaded the region between the two arrows so a thick microtubule bundle formed. (18.0 min) The small growth cone has moved away from the end of the microtubule bundle. (19.5 min) Several microtubules infiltrate the base of the growth cone, beginning the cycle of bundle elongation again. The asterisk marks a constant position in the field. In this case, the asterisks, which marks a position in the growth cone at 0 min, marks the new bend in the axon at 14.5 min and onward. Bar, 5 μ m.

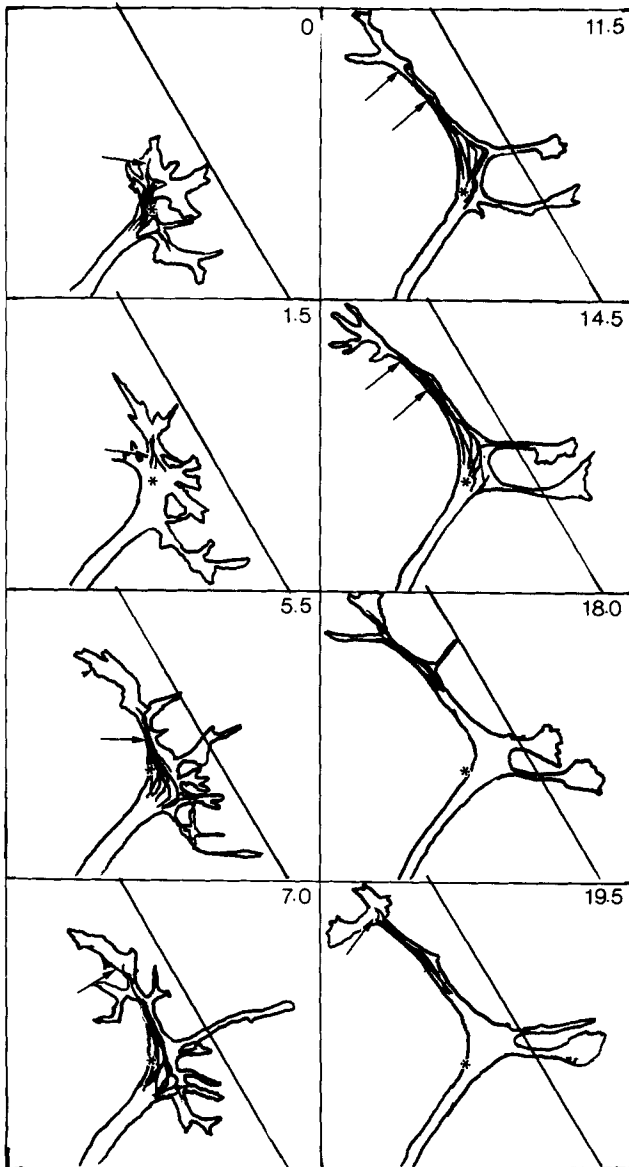
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Figure 4.

the border (*asterisk*). From 7.0 to 19.5 min the microtubule bundle continued to elongate by the same sequence of events. In contrast to the growth cone in Fig. 3, the bending of the microtubule bundle occurs in the growth cone so that the bend in the axon at 14.5 min and onwards actually passes close to the spot where the growth cone approached the border so an asterisk which marks a point on the field coinciding with the central region of the growth cone at 0 min coincides with the bend in the axon at 14.5 min and onwards.

Fig. 5 illustrates microtubules bending toward the new direction of growth, rather than new extension of microtubules into a new direction of growth. The bending of microtubules in Fig. 5 is followed by subsequent invasion of a branch with more microtubules; this branch is eventually transformed into new axon. At 0 min some microtubules in

the growth cone were pointing in the same direction as the axon growing toward the collagen while others had already bent in the direction of the border. The arrow indicates the microtubule ends. By 0.5 min, almost all the microtubules bent laterally so that their terminal portions paralleled the substrate boundary. The top arrow marks the same position in the field as at 0 min while the bottom arrow follows the microtubule ends. At 1.5 min, the bending continued, and the microtubules coalesced into a more cohesive bundle. At 2.0 min, a single microtubule (*arrow*) entered the branch parallel to the border (*arrowheads*). This branch expanded and formed wider veils at its end at 2.0 and 2.5 min so that it now resembled a growth cone. By 3.5 min, multiple microtubules entered the region after the single pioneer (*arrow*). This invasion continued beyond the original area explored by the single microtubule. The original bending of the growth cone microtubules at 0.5 min was preserved in the axonal bend at 3.5 min.

Invasion of Lamella by Individual Microtubules Precedes Consolidation

We observed that microtubules entered the bases of lamella that had spread either just on Matrigel or across the border onto collagen. Out of 25 lamellipodial protrusions each on Matrigel or collagen, 40% and 28% of the lamellae were invaded by at least one microtubule during their life span. Since lamellae extending onto collagen are never ultimately stabilized, this indicates that invasion of a lamella by microtubules does not confer stability to a lamella.

During growth-mediated reorientation, one or a few individual microtubules emerged out of the central bundle and penetrated a lateral lamella or branch before the rest of the microtubules invaded that area (Fig. 5, 0 min, and Fig. 5, 1.5, 2 min). In all six cases where we could clearly resolve single microtubules, single microtubules or very small bundles preceded the invasion by the microtubule bundle. In the other three cases, we lacked single microtubule resolution.

Discussion

In this paper we have investigated the role that microtubules play in growth cone turning. Since microtubules are a major structural component of both axons and growth cones, the organization of microtubules during turning is likely to be an important step in this response. We have studied growth cone turning using cultured neurons encountering a substrate border, because unlike the *in vivo* systems developed so far, it affords an opportunity to visualize turning with high temporal and spatial resolution of both the growth cone shape and the microtubule arrangements. Our major conclusions are that growth cones display heterogeneous behaviors during turning decisions at substrate borders. These behaviors vary between two extremes—one in which the motility of the growth cone dominates, so turning is accomplished by the growth cone tugging on the axon, and to the second, where turning is ultimately linked with the laying down of new axon. The type of turning behavior that occurs and the extent to which microtubules play a role in the turning decision appears to be related to the adhesiveness of the growth cone to the substrate.

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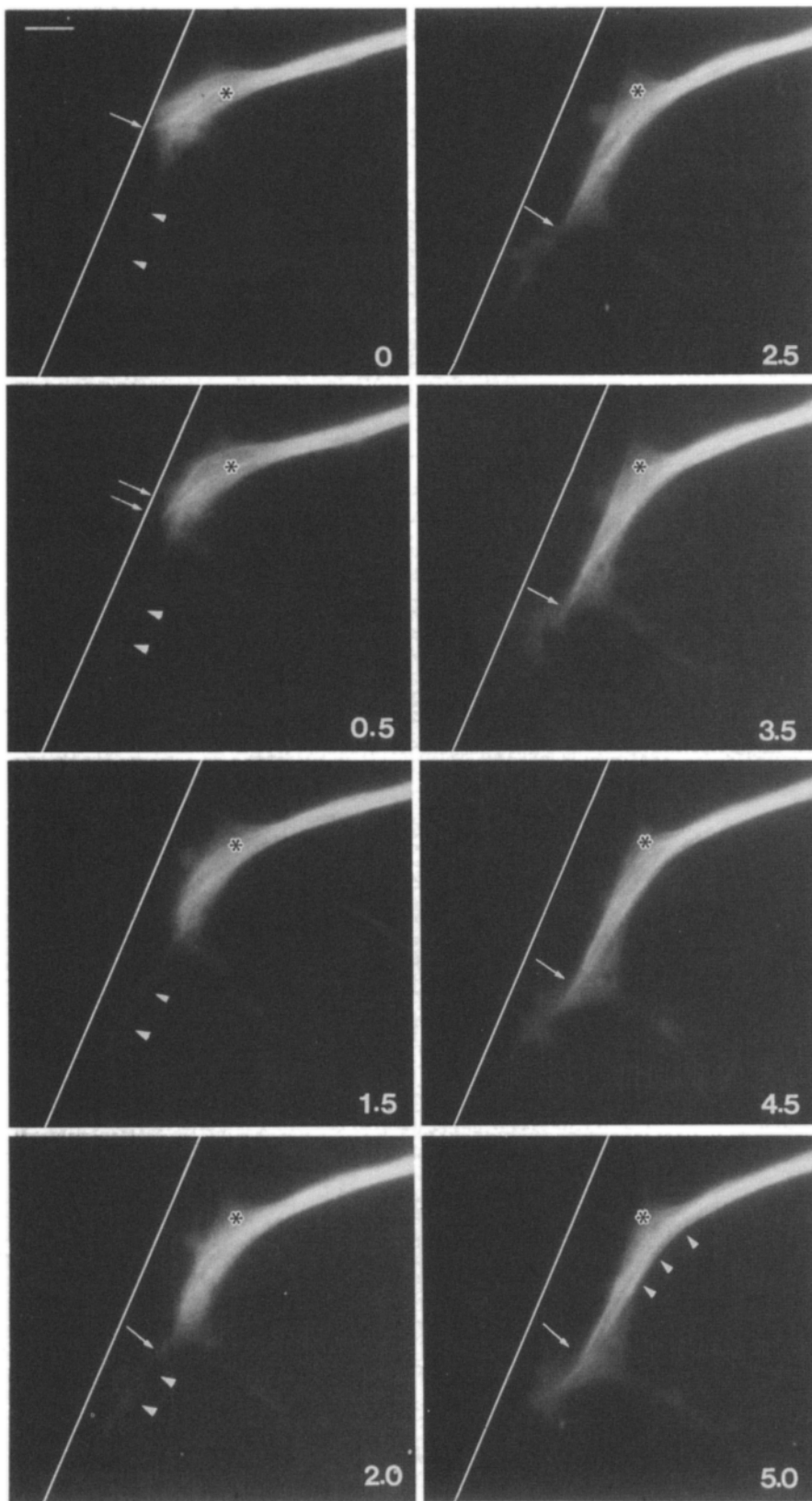


Figure 5. Growth cone microtubules bend toward new direction of growth. (A) Fluorescence images of a turning growth cone and (B) matching traces of the growth cone shape and microtubules. (0 min) The growth cone grew to the border and extended a branch laterally (arrowheads). Most microtubules remained straight, pointing in the direction of the axon while some have already bent to parallel the border. (0.5 min) The lateral branch remained stable, and all growth cone microtubules bent toward it. The upper arrow marks the same point in field as at 0 min. The lower arrow marks the new position of microtubule ends. (1.5 min) The base of the branch dilated with microtubules invading it. The distal portion of the branch (arrowheads) has widened and assumed some protrusive activity. (2.0 min) The stable lateral branch expanded further (arrowheads) and a single microtubule infiltrated the base of the branch (arrow). No microtubules invaded the branch on the right. (2.5 min) The base of the branch widened with more microtubule ends invading it, while the right branch remained free of microtubules. (arrow in 2.0, 2.5, 3.0, 4.5, and 5.0 min mark the same point in the field). At the point where the growth cone originally approached the border, a discrete bend in the axon formed due to the bending and extrusion of microtubules. (3.5 and 4.5 min) The distal area (arrow) was buttressed by the arrival of more microtubules. (5.0 min) Several single microtubules emerged beyond the area marked by the arrow, infiltrating the new growth cone that formed. Microtubules at the bend (arrowheads) clearly form an axonal bundle. The asterisk which marks a constant position in the field marks the growth cone neck at 0 min. By 2.5 min the bending and growth of the microtubule array has transformed this into the axonal bend. Bar, 5 μ m.

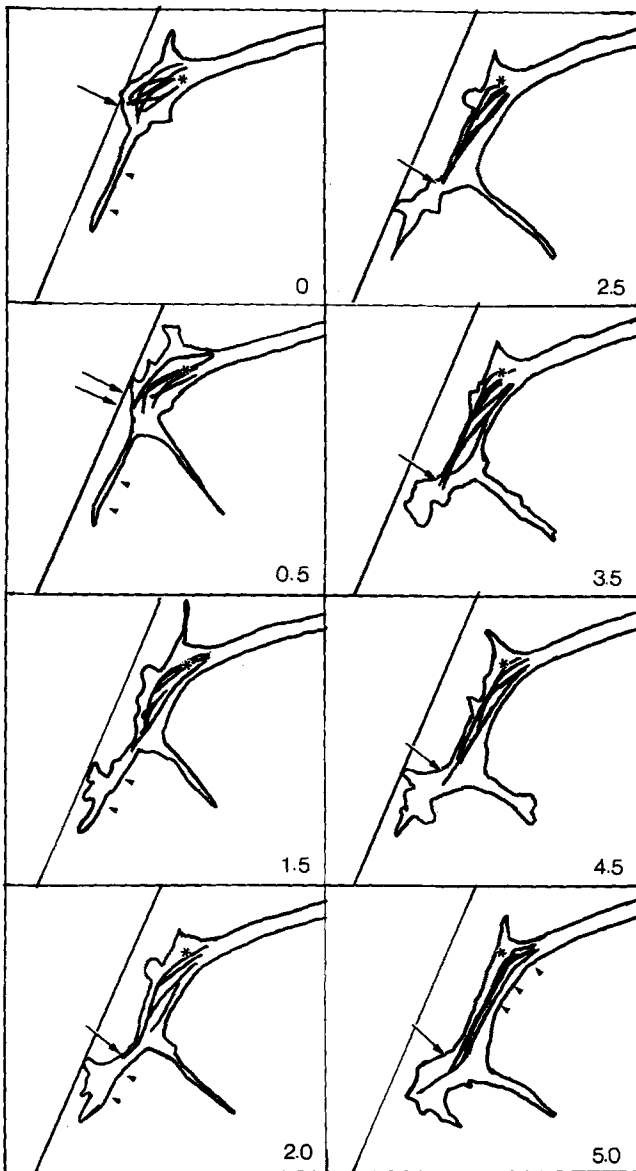
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Figure 5.

The Heterogeneity of Growth Cone and Microtubule Behavior at Substrate Boundaries

When we observed growth cones encountering a substrate boundary, we saw six types of behavior, ranging from growing through the border to turning. While the two neurons that grew through the border both approached the border at ninety degrees, suggesting that neurons may not be able to turn at very steep angles, the other behaviors did not correlate with the angle of approach. The propensity of neurons growing perpendicularly to substrate borders to grow through has also been seen with *Aplysia* neurons (4).

The source of the heterogeneous behavior is uncertain but seems to be most related to the adhesivity of the axon and the growth cone to the substrate. Despite our efforts to apply the Matrigel substrate evenly, the resulting substrate is not perfectly uniform, as inferred from the variable fluorescence

intensity of the substrate in Fig. 1 A. It seems plausible that local concentrations of substrate may affect the adhesiveness and behavior of the growth cone. The neural tube culture is also heterogeneous. Though it is likely to consist mostly of motor neurons, it may not be a pure population. We surmise that a variability of neuronal types may also contribute to the differences in behavior. There may also be an intrinsic variability in the substrate and neuronal behavior *in vivo*. For example, during guidance of a single type of neuron, the T11 pioneer neuron in the grasshopper limb, some variability of behavior is seen from preparation to preparation, particularly at the segment border (17, 19). This suggests that even under the most natural circumstances, there may be some flexibility in the way in which a neuron responds to turning cues.

Among the various turning behaviors of the frog neurons, we did not observe the formation of mature branches with subsequent "micropruning" as was seen for *Aplysia* neurons at a polylysine/glass border and in grasshopper T11 neurons at the Tr-Cx boundary (4, 17). We observed lamella spreading across the border onto collagen which occasionally contained some microtubules. However, in these cases, though the growth cones assumed transient branching configurations, they never formed mature branches reaching across the border. It may be that in the *Aplysia* case, the difference in the glass vs polylysine decision was a more subtle one than the collagen and Matrigel decision for frog neurons. Neurons may also adhere to polylysine by different mechanisms than to the extracellular matrix.

We observed three types of behavior at the substrate border in which the growth cone changed its direction of movement in response to the border. The differences in these behaviors appear to stem from the extent to which the growth cone and the axon adheres to the substrate. In sidestepping, since both the axon and the central region of the growth cone do not remain adherent to the substrate, the lateral movement of the growth cone along the border drags the axon laterally so that no bend is formed in the axon (Fig. 2). This behavior is not informative about growth cone turning, since it does not result in a major reorientation of axonal growth. Furthermore, the relevance of this type of behavior to *in vivo* turning decisions is not clear. It is unlikely that in the three-dimensional context of cells and matrix in the embryo, long axons could remain unattached along their length and be free to translocate laterally through the embryo.

Motility-mediated Reorientation

During motility-mediated reorientation, the growth cone is also not tightly attached to the substrate and can change its direction when the attachment of a dominant lamella causes the growth cone to pull away from the border. With the axon attached to a point near the growth cone, the only structure that could be deformed by the moving growth cone is the segment of axon between the point of attachment and the growth cone (see Fig. 2). The bending of the axon behind the growth cone neck does not appear to require new axonal growth so we feel that in this type of turning, the growth cone acts more like a nongrowing cell that moves over a substrate, rather than a growing neuron. Cultured sea urchin primary mesenchyme cells show very similar behaviors; when a filopodia contacts a bead coated with crude extracellular matrix, the

cell moves toward it (24). Unlike the side-stepping case it seems likely that in vivo, a growth cone could sometimes assume such fibroblastoid qualities, pulling on the axon which maintains substrate attachments just behind the growth cone.

Although we did not directly measure the adherence of growth cone lamellae or the tension generated by the growth cone pulling on the axon, the behavior observed during both side-stepping and motility-related reorientation is consistent with the measurements and observations in other cultured neurons showing that the growth cone pulls and exerts tension on the axon (2, 14). Whereas during neurite outgrowth, this tension is manifested in axonal elongation (7), in the guidance situation studied here, the movement of the growth cone exerts lateral forces on the axon which results in its reorientation.

Various experiments have indicated that the tension produced by the growth cone pulling on the axon is primarily transduced through the actin cytoskeleton (6, 13, 23). Although we have not visualized actin in these experiments, it is clear that during motility-mediated reorientation, the protrusion and retraction as well as the adherence of the actin containing structures such as lamellae dominate the turning decision. In this case, we did not observe a dramatic reorganization of microtubules within the growth cone. The behavior of the microtubules is passive or even irrelevant, since when the entire growth cone moves toward the lamella, the microtubules contained in the growth cone have no choice but to move with it.

Although we have used terms such as the growth cone "pulling away", "tension" and "adhesivity", we do not mean to imply that an adherent lamella exerting tension on the growth cone directly pulls off the other lamellae during the lateral translocations of the growth cone. Our observations cannot distinguish between such a scenario, and one in which growth cone translocation occurs by some lamellae attaining stable attachments to the substrate while other lamellae have unstable attachments and retract.

Growth-mediated Reorientation

During growth-mediated turning, the entire growth cone maintains attachments to the substrate and the entry of microtubules into a lamella appears to be the determining step (Fig. 2). At the collagen border, we observed on several occasions that microtubules within the growth cone bent and elongated in the new direction of growth. The invasion of a lateral lamella by these microtubules predicted the location of new growth cone formation indicating that such exploratory microtubules may be important in choosing the lamella that will eventually be converted into new axon. In this case, the localization of microtubules by dynamic exploration and by microtubule bending and the subsequent elongation of the microtubule bundle are crucial parts of the turning decision. We believe this type of turning closely reflects the types of turning behaviors that occur in vivo, since the axon and growth cone are well attached to the substrate. Indeed, this type of turning resembles the grasshopper T11 neuron where microtubules invade a branch that will eventually become the axon (19).

Many aspects of growth-mediated turning also resemble the characteristics of *Aplysia* bag neurons and PC12 cells growing on highly adherent substrates (1, 8, 15). Even though the *Xenopus* growth cones appear to be much more

dynamic than *Aplysia* neurons grown on polylysine, we see a very similar phenomenon (the extension of the microtubule array into a selected portion of the growth cone, bundling of microtubules, and extension of the microtubule bundle leading to polarized lamellipodium formation).

While the three behaviors we observed—sidestepping, motility-mediated, and growth-mediated turning—may seem like very different responses to guidance cues, the underlying mechanism may actually be similar. In both the sidestepping and motility-driven movement, the key feature seems to be a differential lamellipodial stability and adhesivity that permits the growth cone to avoid the collagen by traversing along the substrate border. The apparently different behavior is due to the constraints imposed by the adhesivity of the axon for the substrate. Whether the axon is straight or bent reflects the mechanical limitation of an intermediate attachment between the cell body and the growth cone. Although the growth-mediated turning seems mechanistically different, it is important to appreciate that these neurons are under further adhesive constraints that preclude the pulling of the axon or even the rapid wandering of the growth cone. In the absence of the permissive distortion of the axon or growth cone we see a localized extension of the growth cone possibly mediated by differential invasion of microtubules. Does the adhesivity of the lamellipodium determine the behavior of the dynamic microtubules that polymerize into them? Or is adhesivity itself a response to an extracellular signal that also promotes microtubule invasion? If the latter is true then the three behaviors of the growth cone during turning are not a reflection of different mechanisms of signal transduction, but instead may be due to differences in the strength or the spatial arrangement of the extracellular cues that induce changes in adhesion and microtubule behavior.

We are deeply grateful to Professor Friedrich Bonhoeffer and Dr. Bernhard Muller for providing the suction apparatus used in constructing the striped substrate pattern, and instructing us in its use. We would also like to thank Professor Louis Reichardt for the use of his fluorescence microscope, Professor John Sedat, Professor David Agard, Dr. Jason Swedlow, and Professor Tim Mitchison for advice on the experiments. We thank Drs. David Drechsel and James Sabry for comments on the manuscript.

Received for publication 15 March 1994 and in revised form 27 October 1994.

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