# The Role of Local Actin Instability in Axon Formation

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**The role of localized instability of the actin network in specifying axonal fate was examined with the use of rat hippocampal neurons in culture. During normal neuronal development, actin dynamics and instability polarized to a single growth cone before axon formation. Consistently, global application of actin-depolymerizing drugs and of the Rho-signaling inactivator toxin B to nonpolarized cells produced neurons with multiple axons. Moreover, disruption of the actin network in one individual growth cone induced its neurite to become the axon. Thus, local instability of the actin network restricted to a single growth cone is a physiological signal specifying neuronal polarization.**

Neuronal polarization occurs when one of the multiple neurites emerging from the cell body elongates rapidly; this neurite becomes the axon, whereas the remaining neurites become dendrites (*1*). What are the intracellular mechanisms specifying this event? Despite intensive analysis, the answer remains elusive (*2*, *3*). Actin assembly and disassembly play an important role in growth cone and leading edge movement (*4*, *5*), in the formation of neurite-like processes in nonneuronal cells (*6*), and in neurite elongation (*4*, *7*). It has been suggested that filopodia of the growth cone create pulling tension needed for neurite elongation (*8*). However, the forces produced by filopodia are not sufficient to translocate the growth cone nor to pull a neurite (*9*), suggesting that forward movement of microtubules produces neurite elongation. Consistently, neurites elongate rapidly and in a disoriented fashion in the presence of cytochalasin D both in vivo and in vitro (*4*, *6*, *7*, *10*). Here, we hypothesized that axon formation takes place from the growth cone with a more dynamic and labile actin network.

Hippocampal neurons form several similar neurites of 10- to 15-μm length before polarization [stage 2 (*1*); see Fig. 1A]. One of the neurites then starts to grow out very rapidly and becomes the axon [stage 3 (*1*, *2*)]. To examine our hypothesis, we analyzed the dynamics of the growth cones of nonpolarized (stage 2) hippocampal neurons in culture until they became polarized (stage 3) using video microscopy (*11*) (Fig. 1). In four out of five analyzed cells, one of the growth cones appeared larger (*3*) and especially dynamic; it extended and retracted numerous lamellipodia and filopodia and also showed turning behavior (Fig. 1B). In contrast to the behavior of this growth cone, the other growth cones appeared smaller and quiescent (Fig. 1B). The neurite with the most active growth cone became the cell's axon (Fig. 1A) (*12*). Because high actin turnover underlies the motility of the growth cones of neurites and also of advancing motile nonneuronal cells (*4*, *5*), we estimated that the observed high dynamics of a single growth cone preceding axon formation reflects higher actin turnover and may underlie neuronal polarization.

If the actin cytoskeleton of the axonal growth cone allows process elongation because it is more permissive for microtubule protrusion than in the other growth cones, a short treatment with cytochalasin D should depolymerize the actin cytoskeleton faster in this growth cone. To test this hypothesis, we treated cells with 1 μM cytochalasin D for 1 min, fixed them, and stained them with rhodamine-conjugated phalloidin (*13*). In polarized stage 3 neurons, the short exposure to cytochalasin D produced a loss of actin staining in the axonal growth cone compared with the minor neurites' growth cones in 72.8% (±7.4%) of the cells (Fig. 2, B and D; $n$ = 214). Consistent with a role in polarization, in nonpolarized stage 2 cells, actin loss after cytochalasin D treatment was restricted to one or two growth cones in 34.2% (±2.1%) of the cells (Fig. 2, A and C; $n$ = 177).

Besides differences in actin susceptibility to cytochalasin D, we also observed differences in the extractability of the growth cones' actin filaments in untreated neurons. We added saponin (20 to 40 μg/ml) to living cells for 10 to 30 s, fixed them, and stained them with rhodamine-conjugated phalloidin (*14*). The loss of actin staining was evident in the growth cone of the axon in 74.1%

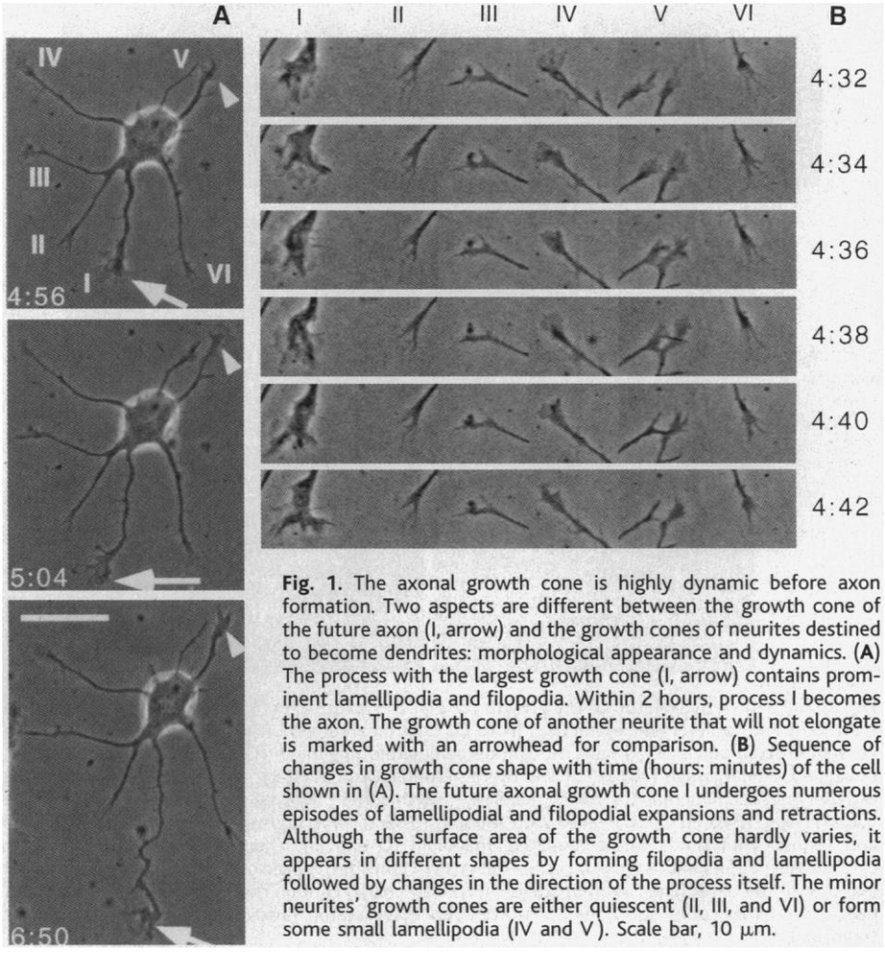


**Fig. 1.** The axonal growth cone is highly dynamic before axon formation. Two aspects are different between the growth cone of the future axon (I, arrow) and the growth cones of neurites destined to become dendrites: morphological appearance and dynamics. (**A**) The process with the largest growth cone (I, arrow) contains prominent lamellipodia and filopodia. Within 2 hours, process I becomes the axon. The growth cone of another neurite that will not elongate is marked with an arrowhead for comparison. (**B**) Sequence of changes in growth cone shape with time (hours: minutes) of the cell shown in (A). The future axonal growth cone I undergoes numerous episodes of lamellipodial and filopodial expansions and retractions. Although the surface area of the growth cone hardly varies, it appears in different shapes by forming filopodia and lamellipodia followed by changes in the direction of the process itself. The minor neurites' growth cones are either quiescent (II, III, and VI) or form some small lamellipodia (IV and V). Scale bar, 10 μm.

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(±2.0%) of polarized stage 3 cells (Fig. 2, F and H; $n$ = 89) and in only one or two processes of 45.2% (±10.0%) of morphologically unpolarized stage 2 neurons (Fig. 2, E and G; $n$ = 124).

The high percentage of stage 3 neurons that lost the actin network in the axonal growth cone only, either after a brief treatment with cytochalasin D or after exposure to a low concentration of saponin, suggests that the axonal growth cone contains an unstable actin cytoskeleton that supports axonal elongation. Following the same rationale, actin instability in a growth cone of a single neurite within a population of stage 2 cells indicates that this is the neurite that will become the axon. In the stage 2 cell shown (Fig. 2, E and G), loss of actin filaments is clear in two growth cones, suggesting that these are the processes with higher chances to become the axon (*15*). The lower percentage of stage 2 cells showing a polarized actin loss compared with stage 3 cells is consistent with the model that hippocampal polarization occurs in a late phase of stage 2 development (*3*).

If actin instability in one growth cone is essential for axon formation, it follows that depolymerization of the actin cytoskeleton in all growth cones of stage 2 cells should produce many axons. To test this hypothesis, we added cytochalasin D for 24 hours to cultures composed primarily of nonpolarized stage 2 cells and then analyzed the neuronal phenotype. This treatment resulted in the appearance of 70.6% (±5.3%) (±standard error) of cells ($n$ = 610) with multiple (two to five) 40-μm or longer processes (Fig. 3A). Addition of dimethyl sulfoxide (DMSO) to sister cultures did not affect the neuronal phenotype, and more than 85% of the cells ($n$ = 611) had the typical stage 3 appearance, one long axon and several short neurites (Fig. 3B). To rule out that the formation of multiple axonlike processes was due to a specific cell-substratum (poly-L-lysine) interaction in our culture system, we analyzed the effect of cytochalasin D on neurons grown on laminin-coated cover slips. The results were identical to those reported above [Web Fig. A (all Web figures are available at www.sciencemag.org/feature/data/987001.shl)]. To certify that the observed disturbance of polarity was due to actin depolymerization and not derived from other possible effects of cytochalasin D, we also depolymerized the actin cytoskeleton with the G-actin–sequestering drug latrunculin B (*16*). Addition of latrunculin B to 1-day-old cells for 24 hours resulted in the appearance of 72.0% (±1.6%, $n$ = 627) of cells with multiple axonlike processes, typically three to four of them (*17*). To test whether the cytochalasin- or latrunculin-induced processes had more axonal characteristics than morphological appearance, we used time-lapse video microscopy to measure elongation rate (Fig. 3E). Addition of 1 μM cytochalasin D resulted in the collapse of lamellipodia and filopodia after 40 to 60 s and protrusion of microtubules into peripheral areas of all of the growth cones (*18*) (Web Fig. B). Then the neurites started to grow and elongated synchronously at an average speed of 4 to 12 μm/hour (Fig. 3E). The measured velocities were identical to those described for axonal growth of untreated hippocampal neurons (*1*). The multiple fast growing axon behavior was observed repeatedly (38 out of 40 cells showed this phenotype, and only two cells grew a single axon). Besides axonlike morphology and growth rate, cytochalasin D–formed neurites contained numerous membrane organelles, a characteristic of the growing axon of control neurons (*3*) (Web Fig. C), and also the dephosphorylated form of Tau, an axonal marker (*19*, *20*).

To demonstrate that destabilization of the actin cytoskeleton in the growth cone is sufficient to induce axon formation, we depolymerized the actin cytoskeleton in selected growth cones. We located individual, unpolarized stage 2 cells, photographed them, and then applied cytochalasin D to a single growth cone for 15 to 30 min with a two-capillary method (*21*). The cells were then returned to the original medium and photographed 24 hours later. In a stage 2 cell shortly before polarization (Fig. 4A), one process had the biggest growth cone and phase-dense material of its shaft and was likely to become the axon (*3*). When the drug was applied to the growth cone of a different neurite, phase-dense material started to collect along the perfused shaft (Fig. 4, B and

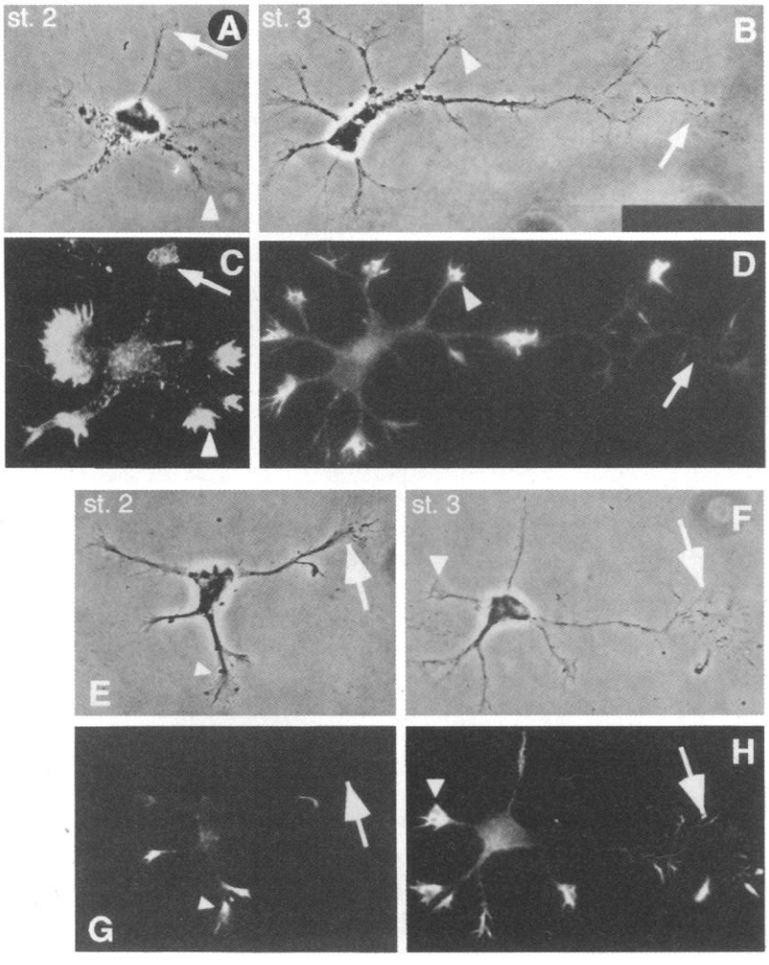


**Fig. 2.** The actin cytoskeleton of the growth cone of the future axon is sensitive to cytochalasin D treatment and to detergent extraction. (**A** to **D**) Cells treated with 1 μM cytochalasin D for 30 to 60 s were fixed and stained with rhodamine-conjugated phalloidin (C and D). The F-actin in the presumed axonal [stage (st.) 2 (C)] and the determined axonal [stage 3 (D)] growth cone (arrow) is depolymerized, whereas the minor neurites' growth cones (arrowhead) still have an intact actin cytoskeleton. (**E** to **H**) Cells treated with saponin (0.02 to 0.04 mg/ml) for 10 to 30 s were fixed and stained with rhodamine-conjugated phalloidin (G and H). The extractability of the actin cytoskeleton differs in the growth cones. The growth cone of the predicted axon in stage 2 cells (G) and that of the determined axon of stage 3 (H) cells (arrow) contain no F-actin, whereas the minor neurites' growth cones still have an intact actin cytoskeleton (arrowhead) after the detergent treatment.

C). One day later, this neurite became the axon (Fig. 4D). From 23 successful observations (cells were still alive 24 hours after the experimental manipulation and grew an axon), 18 neurons grew an axon from the cytochalasin D–perfused site (Web Fig. D). Thus, local actin instability can trigger neuronal polarity.

To demonstrate whether a physiological signaling pathway could produce the same effects on neuronal polarization as observed with actin-depolymerizing drugs, we analyzed the effect of inactivating the Rho family of guanosine triphosphatases (GTPases) (Rho, Rac, and Cdc-42). These proteins were originally identified as polarity-establishing genes in yeast (*22*) and later shown to regulate the actin cytoskeleton in mammalian cells (*23*). Unpolarized hippocampal neurons were treated with toxin B (*24*) for 24 hours, and process formation and integrity of the actin network were then analyzed by light microscopy (Fig. 4, E and F). Treatment with toxin B (100 pg/ml) resulted in the complete loss of F-actin staining (Fig. 4F), and, more importantly, cells showed multiple axonlike processes in 90.6% (±1.8%) of the cases ($n$ = 574) instead of the normal phenotype single axon and multiple dendrites (Fig. 4E). All of the neurites grew extensively, showing lengths ranging from 40 to 80 μm, typical of axons of untreated neurons, and also contained the axonal protein Tau (*20*). Because inactivation of Rac, Rho, and Cdc-42 produced the same effect as addition of cytochalasin D and latrunculin B, with a loss of F-actin and the growth of multiple axons, we conclude that depolymerization of growth cone actin is a

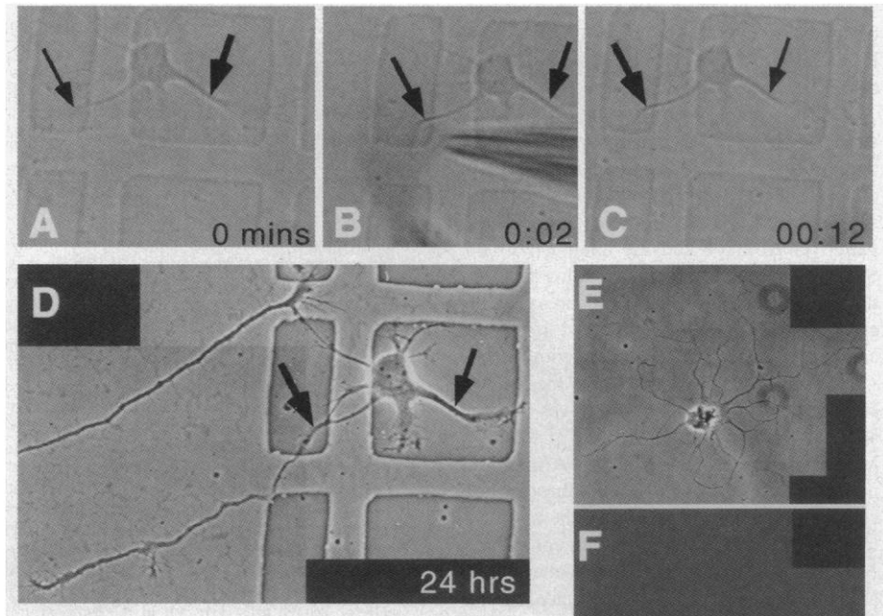


**Fig. 4.** Specific manipulation of the actin cytoskeleton. (**A** to **D**) Axon formation can be artificially determined. One growth cone of a 1-day-old cell was locally perfused with 1 μM cytochalasin D for 12 min (B); the cell was returned to the incubator, and pictures were taken after 24 hours (D). Note that before treatment, the perfused growth cone [(A), thin arrow, left process] is small and with little phase-dense material in its shaft compared with the large growth cone and abundant phase-dense material of the neurite to its right [(A), thick arrow]. This indicates that in this cell, if undisturbed, the latter neurite is destined to become the cell's axon (*3*). Nevertheless, shortly after perfusion, the treated neurite becomes more phase dense (C) and forms the axon during the next 24 hours (D). (**E** and **F**) Toxin B–treated neurons. Cells were treated with toxin B for 24 hours, fixed, and stained with rhodamine-phalloidin (F). The cells are depleted of actin filaments (F) and show many neurites that are 40 to 80 μm long (E). The phenotype is similar to cytochalasin D and latrunculin B treatment (see Fig. 3).

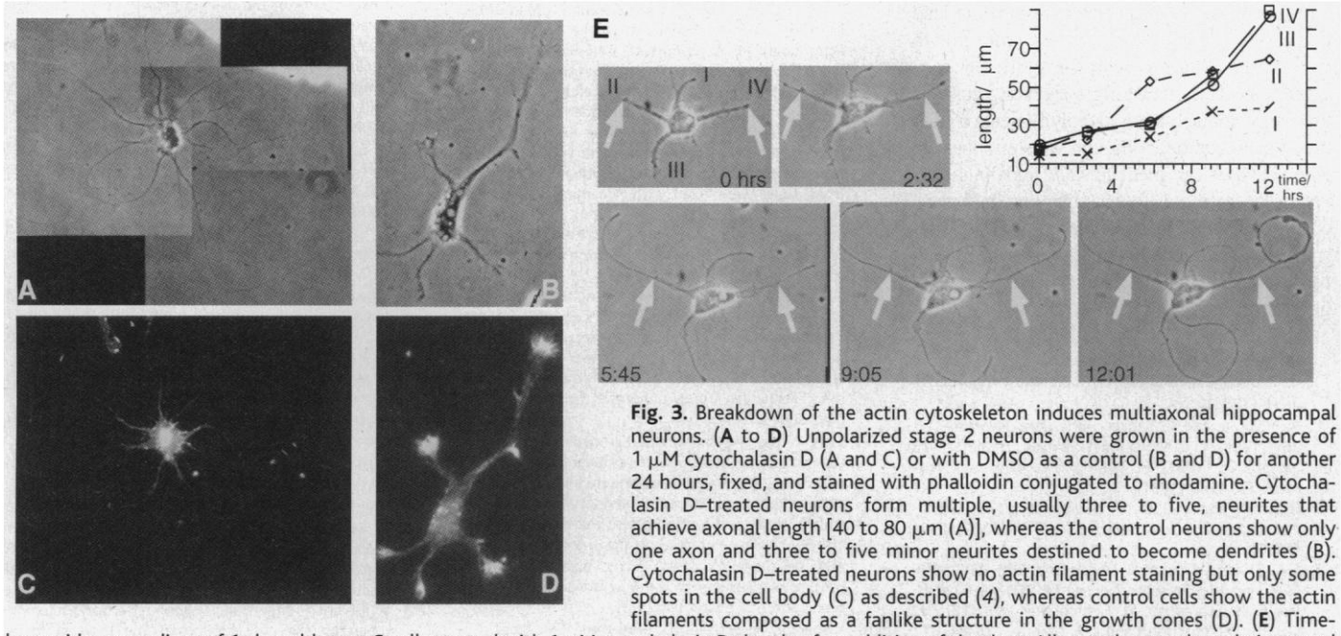


**Fig. 3.** Breakdown of the actin cytoskeleton induces multiaxonal hippocampal neurons. (**A** to **D**) Unpolarized stage 2 neurons were grown in the presence of 1 μM cytochalasin D (A and C) or with DMSO as a control (B and D) for another 24 hours, fixed, and stained with phalloidin conjugated to rhodamine. Cytochalasin D–treated neurons form multiple, usually three to five, neurites that achieve axonal length [40 to 80 μm (A)], whereas the control neurons show only one axon and three to five minor neurites destined to become dendrites (B). Cytochalasin D–treated neurons show no actin filament staining but only some spots in the cell body (C) as described (*4*), whereas control cells show the actin filaments composed as a fanlike structure in the growth cones (D). (**E**) Time-lapse video recordings of 1-day-old stage 2 cells treated with 1 μM cytochalasin D shortly after addition of the drug. All growth cones lose their normal appearance (see also Fig. 1A and Web Fig. B). After 2 hours, the majority of neurites rapidly started to grow. After 12 hours, three neurites are longer than 60 μm, and one neurite is longer than 30 μm. The graph shows the neuritic length over time (hours: minutes). The neurites achieve maximal velocities of 12 μm/hour.

physiological requirement for neuronal polarization. Moreover, these results argue that the Rho family of GTPases is involved in the control of neuronal polarization.

Our data imply that neuronal cells specify axonal and dendritic fate by modifying the dynamics of the actin filaments in the growth cones. In the dynamic growth cone, where the actin network is more unstable, process elongation takes place and the axon is formed. Such a loose actin meshwork allows microtubules to protrude into distal areas of the growth cone, and therefore axon formation can take place (*4*). Consistent with such a view, microtubules invade into the growth cone before the growth cone moves forward and net neurite elongation takes place (*25*). Moreover, beads coated with apCAM, an immunoglobulin superfamily cell adhesion molecule, induce rearrangement of the actin cytoskeleton when bound to growth cones (*26*). Nevertheless, other microtubule-actin interactions besides steric hindrance may regulate microtubules and, hence, process elongation.

In contrast to what happens to induce axon formation, stable actin meshwork in the remaining growth cones may impair microtubule and organelle protrusion. Thus, it may prevent these processes from growing and determine their fate to become "dendrites." It is likely that later dendritic growth will also require a change in actin dynamics. Indeed, dominant negative mutants of Rho, Rac, and Cdc-42 specifically change dendritic formation in cortical neurons (*27*). However, the external signal must differ from that determining axonal growth and is likely to proceed through different members of the Rho family of GTPases (*28*). One may envision that these mechanisms also could lead to neuronal polarization in vivo. One growth cone could contact a factor (or a different concentration of it), activating a signaling cascade that may induce actin depolymerization and process elongation. Although we show that the Rho GTPases can mediate such signaling, activation of many different signaling pathways changes actin filament dynamics. Thus, irrespective of which factor any given neuron contacts, localized actin instability could provide a general mechanism for polarization.

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11. Hippocampal neurons were prepared from 18-day-old embryos as described before [M. De Hoop, L. Meyn, C. G. Dotti, in *Cell Biology: A Laboratory Handbook*, J. E. Celis, Ed. (Academic Press, San Diego, CA, ed. 2, 1998), pp. 154–163)]. The cells were plated onto poly-L-lysine–coated cover slips, either 20 mm in diameter for immunocytochemistry or 40 mm in diameter for long-term observations, or onto gridded cover slips (cellocate; Eppendorf, Hamburg, Germany) for the perfusion experiments. In some experiments, cells were plated on cover slips that were either first coated with poly-L-lysine and then laminin (Gibco-BRL) or with poly-L-ornithine (Sigma) and then laminin. For long-term observations, cells were handled as described [F. Bradke and C. G. Dotti, in *Microinjection and Transgenesis: Strategies and Protocols*, A. Cid-Arregui and A. Garcia-Carrancá, Eds. (Springer-Verlag, Heidelberg, Germany, 1997), pp. 81–94]. Briefly, cells were put in the temperature-controlled FCS-2 long-term observation chamber (Bioptechs, Butler) and positioned on the microscope stage of an inverted microscope (Zeiss, Jena, Germany) equipped with a ×63 Plan-Apochromat objective. Pictures of a chosen cell were taken in 2-min time intervals, with a video camera controlled by image acquisition software (NIH 1.58, Bethesda, MD) to study growth cone dynamics. Longer intervals were chosen for the single-cell observation of cytochalasin D–treated cells. An electronic shutter controlled the light path of the microscope to minimize radiation. Primary pictures were further processed with Adobe Photoshop 3.0 and Canvas 3.5 software.
12. In the fifth cell, one of the small growth cones was the most dynamic and became the axon. The different dynamic properties of the growth cones coincided with the fate of the neurites in all of the observed cases.
13. Neurons were treated with 0.1 to 1 μM cytochalasin D (Sigma), kept as a stock solution of 5 mM in DMSO at −20°C. The alkaloid binds to the barbed end of actin fibers [J. A. Cooper, *J. Cell Biol.* **105**, 1473 (1987)]. In young hippocampal neurons, cytochalasin D depolymerized the actin cytoskeleton completely within 4 min (*20*). The drug was added to the original medium, and cells were then either fixed after 30 to 60 s to partially depolymerize the cytoskeleton or returned to the incubator for another day and then fixed. Cells were fixed with 4% paraformaldehyde containing 4% sucrose at 37°C for 12 min, quenched in 50 mM ammonium chloride, and extracted in 0.1% Triton X-100 for 2 min. After blocking with a solution containing 2% fetal bovine serum, 2% bovine serum albumin, and 0.2% fish gelatine dissolved in phosphate-buffered saline, cells were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Leiden, Netherlands), which was stored as a methanol stock solution at −20°C, at room temperature for 30 min. Latrunculin B was purchased from Calbiochem (Bad Soden, Germany).
14. Cells were detergent extracted in medium equilibrated to air concentration of gases containing saponin (0.02 to 0.04 mg/ml) at room temperature for 10 to 30 s and immediately fixed. Standard filter sets (Zeiss) for rhodamine were used for fluorescent microscopy.
15. The final decision of which of the processes elongates may be made by other events also essential for process elongation: microtubule advance and membrane addition as discussed (*3*).
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17. Although the effect in neuronal phenotype was clear, we also performed single-cell analysis to establish whether these cells derived from truly unpolarized neurons. Stage 2 cells grown on gridded cover slips were localized on the microscope stage, photographed, and then placed in medium containing 1 μM cytochalasin D or DMSO for 24 hours. In 77.6% (±3.7%) of the cytochalasin D–treated neurons ($n$ = 138), the cells showed two or more long processes, whereas in DMSO-treated cells, only 11.6% (±1.3%; $n$ = 50) showed a second axon.
18. The cells were fixed 1 min after the first neurite started to grow and stained with mouse antibody to tubulin (Amersham) and visualized with a sheep antibody to mouse conjugated to fluorescein isothiocyanate (Amersham) and phalloidin conjugated to rhodamine. This experiment revealed that microtubules had invaded the distal areas of the growth cones (Web Fig. B II), whereas in control cells the microtubules remained in the central area of the growth cone.
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21. We built a device to locally perfuse cells in the following way. Two micropipette holders were installed on an inverted microscope to form an angle of 60° between them. One pipette holder kept the glass needle containing the applied drug in position, whereas the other pipette holder positioned the sucking glass needle. The drug was dissolved in neuronal medium equilibrated to air concentration of gases. The medium was labeled with cherry red (Brauns-Heitmann, Warburg, Germany) to trace the application of the drug. The ejection needle prepared with a needle puller (Sutter Instruments, Novato, CA) had a tip diameter smaller than 3 μm. The sucking needle tip was around 5 μm in diameter. A peristaltic pump (Instech, Plymouth-Meeting, PA) simultaneously drove the application and sucking of the drug. The velocity of application was minimal to avoid physical disturbance of the cell. Experiments were performed in the following way. Cells grown on a gridded cover slip were put in a tissue culture dish with a glass bottom (MatTek Corporation, Ashland, MA) containing neuronal medium equilibrated to air concentration of gases. After the cells were put on the microscope stage, the glass needles were installed to the pipette holders and localized to the optic field at low magnification. A typical stage 2 cell was chosen, and the glass needles were positioned in close vicinity of the desired growth cone, creating a local field of cytochalasin D on only one growth cone (see Fig. 4). Either 1 μM cytochalasin D or DMSO was applied to one neurite for 15 to 30 min. Cells were constantly recorded to control the perfused area. The cells were then immediately returned into the original medium in the incubator.
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29. We thank K. Aktories for the generous gift of toxin B; C. Sanchez for the MAP-2 antibody; B. Hellias for preparing the cell cultures; T. Hyman, V. Moreau, R. Parton, K. Simons, M. Way, and W. Witke for intense discussions and helpful suggestions on the manuscript; and F. Engert and T. Bonhoeffer for discussions regarding the construction of the local perfusion set-up. C.G.D. is supported by the Sonderforschungsbereich 317.

10 December 1998; accepted 18 February 1999