Mechanical tension can specify axonal fate in hippocampal neurons

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Here we asked whether applied mechanical tension would stimulate undifferentiated minor processes of cultured hippocampal neurons to become axons and whether tension could induce a second axon in an already polarized neuron. Experimental tension applied to minor processes produced extensions that demonstrated axonal character, regardless of the presence of an existing axon. Towed neurites showed a high rate of spontaneous growth cone advance and could continue to grow out for 1–3 d after towing. The developmental course of experimental neurites was found to be similar to that of unmanipulated spontaneous axons. Furthermore, the experimentally elongated neurites showed compartmentation of the axonal markers dephospho-tau and L-1 in towed outgrowth after 24 h. Extension of a second axon from an already polarized neuron does not lead to the loss of the spontaneous axon either immediately or after longer term growth. In addition, we were able to initiate neurites de novo that subsequently acquired axonal character even though spontaneous growth cone advance began while the towed neurite was still no longer than its sibling processes. This suggests that tension rather than the achievement of a critical neurite length determined axonal specification.

Introduction

Cultured hippocampal neurons have been widely used as a model system for the study of axonal and dendritic development (Craig and Banker, 1994). These neurons undergo a highly stereotyped sequence of axonal development. Axons develop from among undifferentiated "minor processes," only one of which typically becomes an axon. Indeed, the rapid outgrowth of one minor process to subsequently become the axon is among the first events in the development of neuronal polarity (Dotti et al., 1988; Craig and Banker, 1994). This suggested to us a possible link between mechanical tension and axonal specification because we have shown that neurite elongation rate is a linear function of the magnitude of pulling tension, provided either by the growth cone (Lamoureux et al., 1989) or by experimental manipulation (Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997). Axonal specification has been discussed recently in terms of events that break the symmetry of the cell (Andersen and Bi, 2000; Bradke and Dotti, 2000), and experimentally applied tension can break symmetry in several types of cultured neurons by initiating neurites de novo from a rounded cell body (Bray, 1984; Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997). In observations of the earliest events of spontaneous axon initiation in chick sympathetic neurons, tension was again strongly implicated as playing a role in symmetry breaking and neurite initiation (Smith, 1994). Previous work on axonal specification in hippocampal neurons has focused on the role of one process attaining a critical length (Dotti and Banker, 1987; Goslin and Banker, 1989) or the role of actin dynamics in the growth cone (Bradke and Dotti, 1999; Ruthel and Hollenbeck, 2000). Both of these aspects of the problem also have links to tension. Neurite lengthening in culture usually involves growth cone advance, which in turn is dependent on the growth cone exerting tension (Lamoureux et al., 1989). The ability of the growth cone to exert tension is widely regarded to depend on the actin cytoskeleton (Lin et al., 1994; Mitchison and Cramer, 1996). Thus, the experimental findings on axonal specification combined with cytomechanical results suggested to us a simple proximate mechanism for determining the axon: the minor process that first exerts sufficient tension to overcome a threshold and begins rapid elongation becomes an axon.

We tested this hypothesis using calibrated glass needles to experimentally apply tension to minor processes of rat

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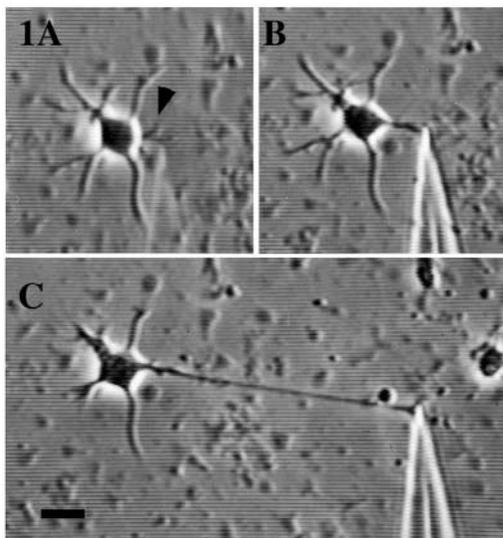
Figure 1. Elongation of a minor process in a stage 2 neuron induced by experimentally applied tension. (A) Neuron immediately before needle application. Arrowhead marks short process to which needle was attached. (B) Same neuron 0:30 h/min later during early stage of neurite towing. (C) 5:40 after panel B, at the end of towing. Bar, 20 μm.

hippocampal neurons. Neurons at two stages of development were used: those in which all neurites were minor processes (stage 2 of Dotti et al., 1988) and neurons that had already specified one process as an axon (stage 3). Minor processes of stage 2 or stage 3 neurons were elongated by "towing," and subsequent identity as an axon was determined using several criteria: rapid rate of spontaneous growth cone advance, capacity of the towed neurite to remain elongated and undergo continued spontaneous elongation, and the presence of early molecular markers for axons.

Results

Minor processes of hippocampal neurons elongate in response to applied tension

Cultured hippocampal neurons were manipulated at two different stages of polarity development. Stage 2 neurons (which have minor processes but no axon) were used to determine whether a particular minor process could be induced to differentiate into an axon by experimentally applied tension. A typical example of the elongation that resulted from manipulating a minor process of a stage 2 neuron is shown in Fig. 1. Fig. 1 illustrates a routine aspect of these manipulations for stage 2 neurons: the minor process for elongation was explicitly chosen to be the least, or among the least, well developed in terms of neurite length. Because a large growth cone has been reported to be indicative of a developing axon (Bradke and Dotti, 1999), some effort was also made to choose the smallest growth cone, although we found that the size of minor process growth cones varied significantly over time (Ruthel and Hollenbeck, 2000). Hippocampal neurons typically elaborate only a single axon, suggesting that after one process has been chosen as the axon the remaining minor processes are inhibited from becoming axons and become destined to differentiate into dendrites. To determine whether mechanical tension can specify an axon in the presence of an existing axon, minor processes of stage 3 neurons were also experimentally lengthened. Fig. 2 shows an example of the experimental elongation of a minor process from a stage 3 neuron. Elongation of a minor process of a stage 3 neuron did not result in the loss of the original axon either during the experimental elongation or during subsequent unassisted outgrowth (Fig. 2 D). Indeed, both the original axon (as branch growth) and the towed neurite continued to exhibit substantial outgrowth after tension was no longer applied.

Like other neuronal types exposed to this experimental growth regime (Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997), tension applied to existing hip-

Figure 2. Elongation of a minor process of a stage 3 neuron induced by experimentally applied tension. (A) Immediately before the beginning of the manipulation, the extant axon was 250 μm long, and the minor process (arrowhead) was 25 μm. (B) 28 min after needle attachment. (C) Immediately after needle removal, the minor process had been towed to a length of 100 μm. (D) After 18 h of incubation following needle detachment, the spontaneous axon had branched and produced a net elongation of 70 μm, whereas the experimental neurite elongated an additional 90 μm. Bar, 50 μm.
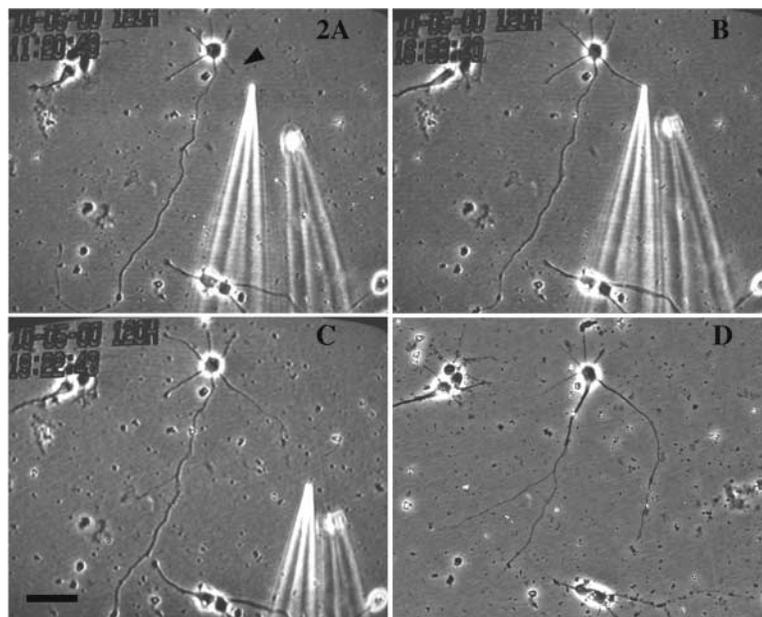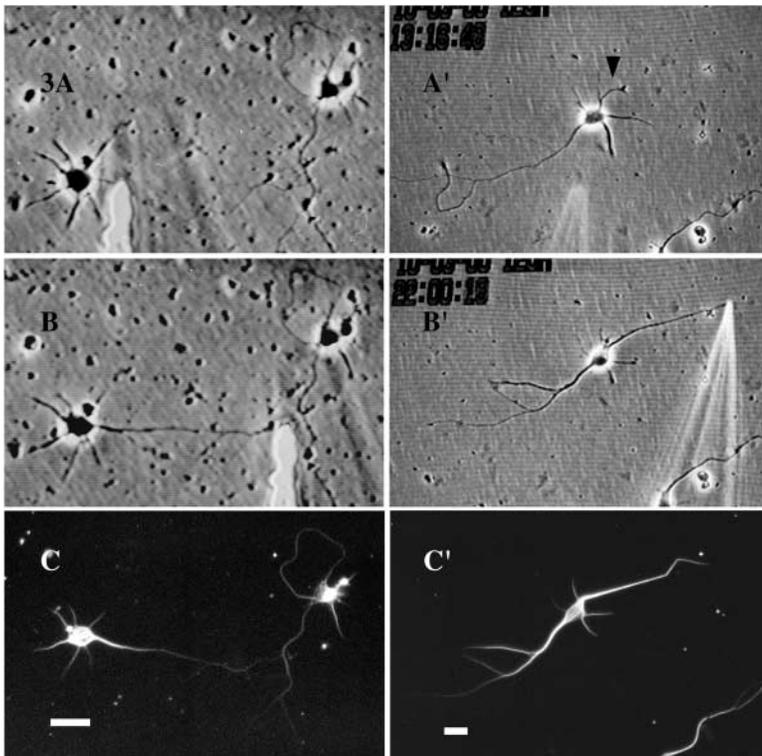
Figure 3. **Elongation by towing produces neurites with a high density array of microtubules in stage 2 and stage 3 neurons.** (A) At the beginning of towing of a stage 2 neuron. (B) At the end of towing 4:30 later. Immediately after needle removal, the cell was fixed, lysed, and processed for microtubule immunofluorescent localization (C). Bar, 20 μm. (A') At the beginning of towing of a stage 3 neuron. (B') At the end of towing ~9 h later. (C') Immediately after needle removal, the cell was fixed, lysed, and processed for microtubule immunofluorescent localization. Bar, 20 μm.

pocampal neurites produced extensive elongation that maintained a uniform caliber (Figs. 1 and 2) and contained a high density array of microtubules (Fig. 3). Also similar to all other cultured neurons investigated, the elongation rate of hippocampal neurites was a robust linear function of experimentally applied tension (Fig. 4). That is, each neurite shows a linear relationship of elongation rate with respect to the tension levels applied to it, but the slopes (tension sensitivity) and x-intercepts (threshold for growth) of the linear relationships differ from cell to cell and vary over a range. For example, the top line in Fig. 4 comes from a stage 3 neuron whose spontaneous axon was pulled, whereas the bottom line is the result of towing a minor process of a stage 2 neuron. The minor processes and axons of hippocampal neurons showed qualitative and quantitative characteristics quite similar to that of chick forebrain neurons (Chada et al., 1997; Heidemann et al., 2001). Like chick forebrain neurons, rat hippocampal neurites have a low threshold, 20–40 μdynes to begin elongation as shown in Fig. 4. Peripheral neurons, in contrast, do not typically elongate their neurites until threshold forces of ~100–200 μdynes are reached (Zheng et al., 1991; Lamoureux et al., 1997). Like chick forebrain neurons, hippocampal neurons show rapid growth (100 μm/h) at tensions of <100 μdynes. Like the two types of neurites shown in Fig. 4, all hippocampal neurites show an increase in growth rate of ~1–3 μm/h for each additional μdyne of tension applied. This degree of growth responsiveness to tension is similar to that found for chick sensory neurons, chick forebrain neurons, and PC12 cells (Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997). Thus, hippocampal neurites, minor processes, or axons grew in response to tension very much as do other cultured neurons (Heidemann et al.,

1995). More generally, we noted no systematic difference in experimental neurite behavior between stage 2 and stage 3 neurons in 1–2-d-old cultures or between axons and minor processes. That is, all neurites behaved over a similar range with respect to ease of needle attachment, forces required for elongation, tendency for detachment while under tow,
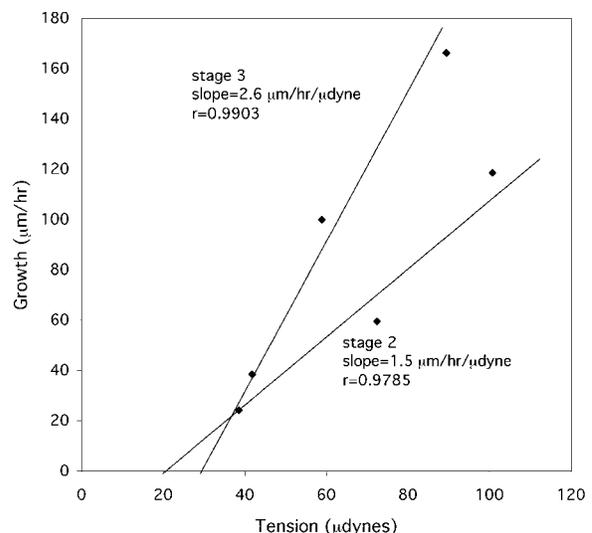
Figure 4. **Growth rate of rat hippocampal neurites as a function of applied tension.** Two neurites from two neurons were subjected to a step-function protocol of experimental tension, where each step (data point) represents towing at constant tension for 60–90 min. Growth rate was calculated from the increased length of the neurites divided by the elapsed time under tow at that tension. The top line was from a spontaneous axon of a stage 3 neuron, and the bottom line was from a minor process of a stage 2 neuron.

Figure 5. **Spontaneous advance of the growth cone of a stage 3 neurite occurring during the towing process.** (A) Experimental neurite (arrowhead) immediately before manipulation. (B–D) Growth cone spontaneously undertakes to change adhesion to the culture dish and extends 48 μm over the next 90 min. Bar, 20 μm.
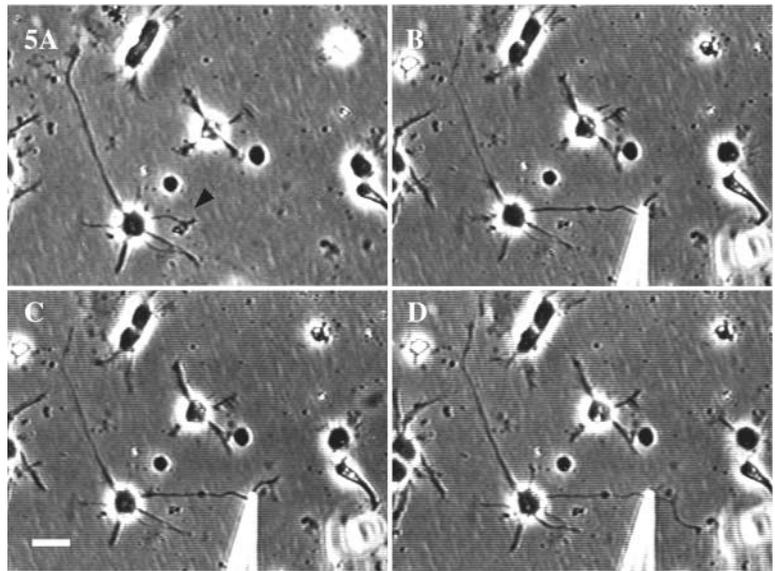
likelihood of spontaneous growth cone advance, and subsequent longer term growth.

In 10 control experiments, we attached a polylysine-ConA–treated needle to a minor process of a stage 2 neuron and did not exert any tension, i.e., no manipulations of the processes after attachment was achieved. In all 10 trials, the experimental minor process failed to respond in any observable manner to attachment only. There were no elongations of the minor processes, no change in motility of their distal ends, and the other unattached, minor processes continued to show their characteristically limited activity. We attempted to maintain attachment of each minor process for 3 h, but in half the cases the minor process changed its attachment from the needle to the dish and "sat there." Among those neurons still observable the following day, none of the experimental minor processes subsequently showed spontaneous elongation into an axon. This unresponsiveness is in sharp contrast to the variety of behaviors stimulated by manipulations of the needle as outlined in the data below. We conclude that attachment and presence of the experimental needle is not itself a growth or motility stimulus to minor processes, rather that tension and deformation is the relevant experimental stimulus imposed by the needle.

### Experimentally elongated neurites show spontaneous rapid growth

Among the earliest indications of spontaneous axon determination in hippocampal neurons is the rapid outgrowth of one minor process (Dotti et al., 1988; Craig and Banker, 1994). During the course of our experiments, we found that neurites that were subjected to alternating periods of tension and relaxation frequently changed their attachment from the needle to the substratum and began elongation guided by its own growth cone motility. Although the growth cone changed attachment and activity to the substratum, the experimental neurite invariably remained attached to the needle, in contrast to the control experiments above. Fig. 5 shows an example of growth past the needle (Fig. 5, B–D) in an early stage 3 neuron. It is noteworthy that rapid growth

past the needle first began when the towed neurite was only 60% the length of the original axon, suggesting that matching the length of the original axon was not necessary for this behavior. Among 50 experimental neurites (27 stage 2; 23 stage 3), we observed 890 μm of spontaneous growth past the needle during a total period of 60 h, comprising individual examples ranging from 5 min to 2 h 20 min. Thus, these serendipitous bouts of spontaneous growth cone advance averaged 15 μm/h, although we observed maximum rates of elongation as high as 80 μm/h, albeit only during a brief 8-min period. More generally, short term maximum growth rates were in the 20–40 μm/h range. During longer term time-lapse observations of axon outgrowth, others reported slower average elongation rates of 6–7 μm/h but similar maximum growth rates (Dotti et al., 1988; Ruthel and Banker, 1999). Our higher average rates may reflect the tendency for initial axon outgrowth to be more rapid and contain fewer pauses than later axon outgrowth (unpublished data). In agreement with Dotti et al. (1988), we observed very little long term change in the lengths of unpulled minor processes (<1 μm/h on average).

Experimentally elongated neurites have the same life histories of outgrowth and persistence as do spontaneous early axons

If experimentally elongated neurites become axons, we would expect them to remain extended and continue elongating in the days after a successful needle removal. In Fig. 2, for example, a minor process of a stage 3 cell was towed and the original axon (left branch) and experimental neurite both showed growth, unlike the remaining minor processes.

Fig. 6 shows an example of extensive spontaneous outgrowth from an experimental neurite of a stage 2 neuron. After successful needle removal (Fig. 6 B), the experimental neurite grew an additional 225 μm during the first 38 h of incubation (Fig. 6 C) and grew a branch 24 h later (Fig. 6 D). Note in these images the near absence in growth of any of the minor processes during this period. After developing the tension-relaxation protocol to encourage growth cone
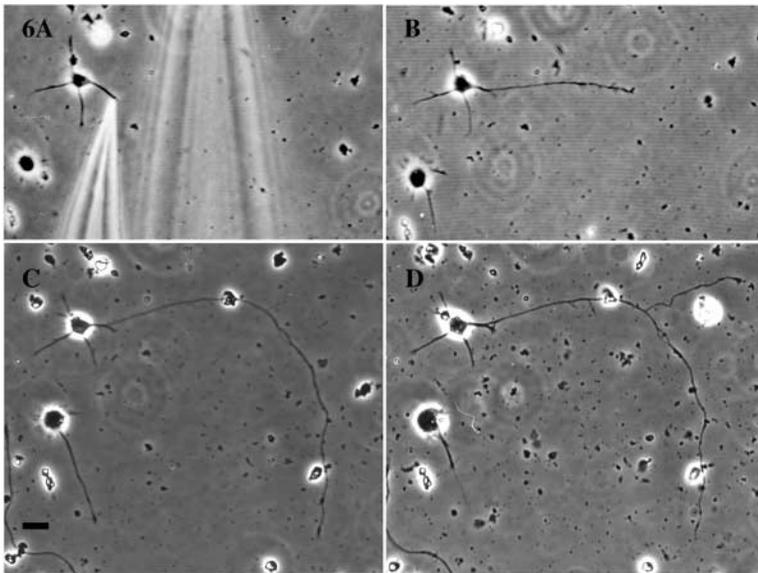
Figure 6. **Longer term spontaneous growth subsequent to towing neurite of stage 2 cell.** (A) Neurite of stage 2 cell immediately after needle attachment. (B) Neuron at end of towing process after needle detachment. (C) Same neuron 38 h after B. The towed neurite has undergone 225 μm of spontaneous elongation. (D) 1 d after C, the towed neurite has now grown a branch. Bar, 20 μm.

activity, as described above, 31 of 38 experimental neurites (20 stage 2 and 11 stage 3 neurons) that were successfully detached from the needle grew or at least remained extended for >24 h after experimental intervention. 23 out of 38 grew at least an additional 50% beyond their towed length; 12 of these 23 more than doubled their towed length. Five more grew slightly (15–25%), whereas three remained extended but did not change length significantly. Only 7 out of 38 neurites failed to persist; generally the towed neurite retracted and no axon formed at all; in two cases a different neurite became an axon.

We wished to determine whether the extent of axonal growth and its stability among experimental neurites was similar to that of spontaneously specified axons at early times in stage 3. We followed the fate of 45 selected neurons that had spontaneously developed to stage 3 (one neurite >75 μm) on the day after plating. Thus, these cells were in culture for the same period and showed a similar extent of development as our experimental neurons, except that here we did not use stage 2 neurons. These controls were subjected to similar insults as experimental neurons (same changes of medium, several hours on the heated microscope stage, same perturbations of the medium incident to transport, etc.) but without needle interventions of any kind. Of these 45 early stage 3 neurons, 30 maintained the initially specified neurite as an axon by remaining extended or growing. Of these, 5 out of 30 remained extended with no additional growth, 5 out of 30 grew slightly, 8 grew more than 50%, and 6 more than doubled their length, whereas 5 grew an apparent second axon, and 1 became tripolar. Importantly, in the remaining 15 cases, the originally specified axon retracted, and a different neurite subsequently became the axon. Thus, the extent of outgrowth and stability of specification among spontaneously initiated axons was similar to that seen among experimentally manipulated neurites. On an anecdotal level, our observations of these control neurons were noteworthy because we saw that unmanipulated axons undergo many of the same growth behaviors that ma-
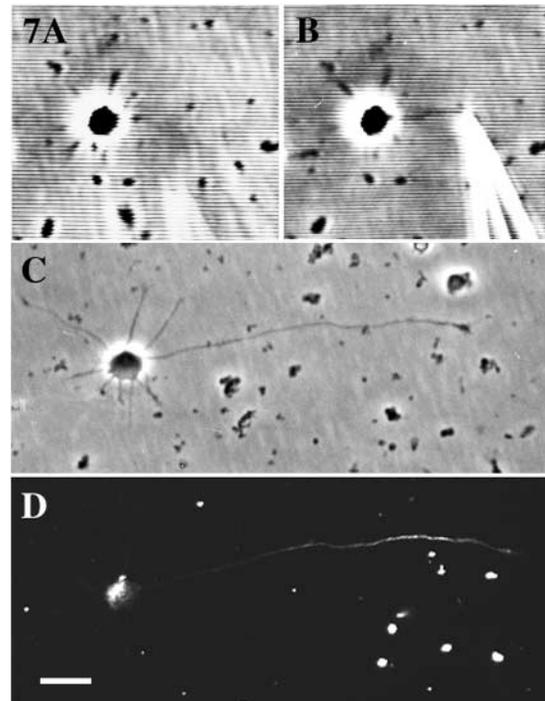
Figure 7. **Immunofluorescent localization of dephospho-tau in experimental neurite of stage 2 neuron after 1 d of incubation.** (A) Needle is attached to one of the smallest minor processes of a stage 2 neuron. (B) After slightly more than 2 h of towing with alternating periods of tension and relaxation (see Results), the experimental neurite has elongated 25 μm. At this point, the growth cone advanced slightly beyond the needle, and this opportunity was taken to remove the needle and the culture replaced into the incubator. (C) By the next day, the experimental neurite had elongated 90 μm. (D) 2 h later, the neurite had elongated an additional 20 μm and was fixed and processed for dephospho-tau localization. As in this example, a clear compartmentation and gradient of dephospho-tau was seen in experimental neurites allowed to continue elongation for 1 d after towing. (Those experimental neurites fixed immediately after towing showed compartmentation but no gradient [not depicted].) Bar, 20 μm.

Figure 8. **Immunofluorescent localization of dephospho-tau in experimental neurite of stage 3 neuron after 1 d of incubation.** (A) Immediately after needle attachment to experimental neurite. (B) At the end of towing, 9 h 43 min later, experimental neurite is 400 μm long. (C and D) Matched phase and fluorescent images of experimental cell fixed after 20 h of incubation after needle removal. Note that the spontaneous axon elongated from 125 μm to 190 μm during these 20 h. The experimental neurite shortened immediately after and incident to needle removal, but then remained essentially stable at the length shown in C and D.
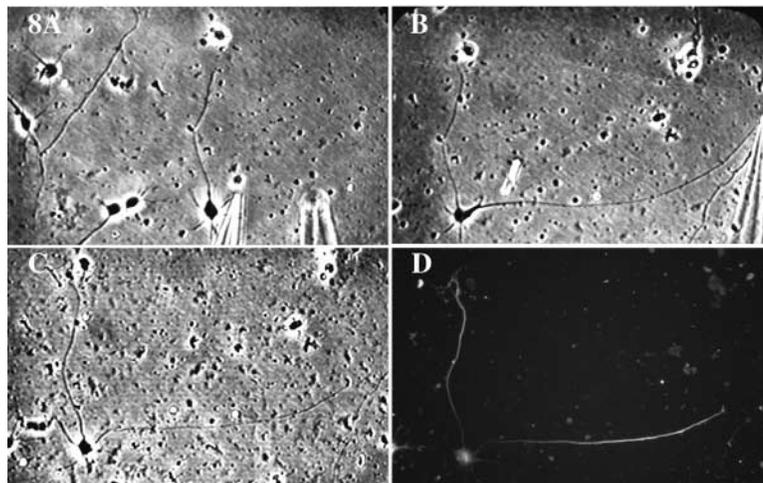
nipulated neurons undergo, including ones we had presumed were artifacts of towing. One example of the latter is that we saw two instances among the 45 in which rapid elongation of the axon was accompanied by the translocation of the somatic cytoplasm and nucleus into the axonal shaft, leaving a still motile "ghost" of the cell body. We had reported this phenomenon previously in brain neurons subjected to hard towing (see Fig. 6 in Ingber et al., 2000). Thus, we were surprised by the similarity of spontaneous and experimental neurites at early times of axonal specification in life course and phenomenology.

Experimentally elongated neurites show molecular markers for axons

An early marker for axons in hippocampal neurons is the enrichment of dephospho-tau in a distal to proximal gradient in the axon (Mandell and Banker, 1996; Ruthel and Hollenbeck, 2000). Among 30 neurons examined, 16 stage 2 and 14 stage 3 neurons, all experimentally elongated neurites examined showed compartmentation of dephospho-tau antigen. All six cells fixed immediately after towing showed compartmentation as a general enrichment of dephospho-tau to the axon, with fairly uniform distribution (data not shown). However, after a day in incubation 16 out of 24 cells showed a clear gradient of dephospho-tau immunofluorescence, whereas the remaining 8 neurons continued to show uniform compartmentation (see Fig. 11). Fig. 7 shows the distal to proximal dephospho-tau gradient seen in the experimental neurite of a stage 2 neuron permitted to grow for an additional 24 h after towing. Fig. 8 shows a similar result for dephospho-tau compartmentation in both the towed neurite and spontaneous axon of a stage 3 neuron after 24 h.

Another molecular marker that is enriched in axons at early times after specification is the cell adhesion molecule, L1 (Van den Pol and Kim, 1993). Fig. 9 shows an example in which a minor process of a stage 2 neurite is elongated for >3 h, then fixed immediately and stained for L1. As shown here, L1 is rapidly compartmentalized to the axon that has been experimentally specified. As reported previously for spontaneous axons of hippocampal neurons (Van den Pol and Kim, 1993), we found that L1 has a punctate distribution along the experimental neurite of our cells.

Even short tension-induced neurites grow and develop as axons

One hypothesis for the mechanism of axonal specification of minor processes is the achievement of a critical length. In this scenario, relative length is a "symmetry breaker" and axonal specification occurs when a minor process becomes ∼15 μm longer than any of its sibling neurites (Goslin and Banker, 1989). As the foregoing data indicate, the very close relationship between tension and length that characterizes neurite outgrowth makes separating the two difficult. How-
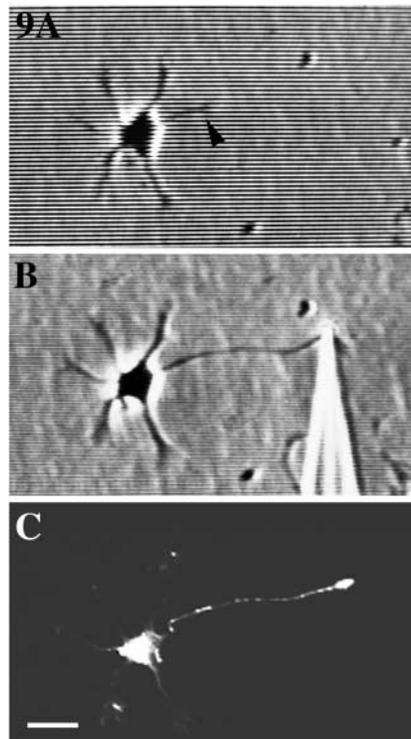
Figure 9. **Immunofluorescent localization of L1 in experimental neurite of stage 3 neuron immediately after towing.** (A) A minor process (arrow) was attached to a needle and elongated ∼50 μm over 3 h and 40 min (B). (C) After needle removal, the cell was fixed and processed for L1 localization. As shown here, the experimental neurite shows the punctate distribution of L1. Bar, 30 μm.
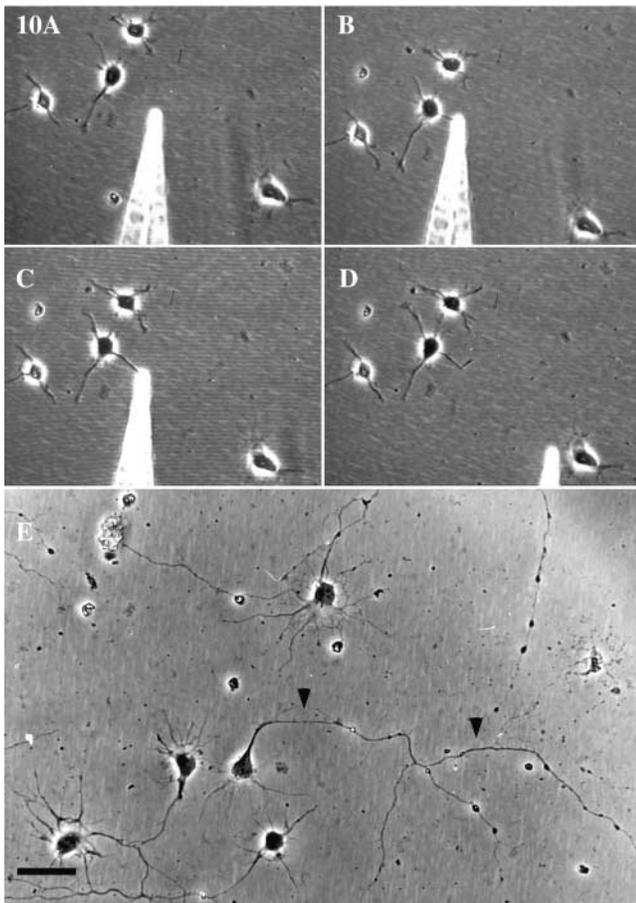
Figure 10. **De novo initiation of short experimental neurite and subsequent spontaneous lengthy elongation during 4 d of incubation.** (A) Neurite of stage 2 cell immediately before needle attachment. Note the presence of landmark cells around the experimental neuron. (B) After the 20 min required for needle attachment and 3 min of applied tension, a short neurite has been initiated. (C) 3 h later at the end of towing, the experimental neurite has elongated 30 μm. Note that the experimental neurite remains shorter than its sibling neurites. (D) Immediately after needle detachment, which slightly elongated and deformed the experimental axon. (E) 3 d after D (and a 90° counterclockwise rotation of the optical field), the experimental neurite (arrowheads) has elongated extensively, whereas the remaining minor processes have remained short. Bar, 50 μm.

ever, we obtained 14 examples, including both stage 2 and stage 3 neurons, where experimental tension caused axonal specification in experimental neurites only towed a short distance, so they were shorter than their sibling minor processes at the end of towing. Most of these came from experiments that exploited the capacity to initiate neurites de novo (and thus begin at zero length) by applying mechanical tension to the cell body. The initiation protocol was similar to that described previously for other cultured neurons (Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997). Attachment of the needle to the margin of the hippocampal cell body and subsequent towing causes initiation of a uniform caliber neurite (Figs. 10 and 11). Given the required delicacy of towing to initiate a neurite, the initiation protocol allowed a 2–3-h period of towing that ceased while the experimental neurite was still shorter than its sibling neurites. Among 14 initiation experiments in which the towed neu-

rite remained shorter than its siblings (and the needle was successfully removed), 5 out of 14 showed no growth of any of the neurites and 9 out of 14 subsequently grew into long, axon-like extensions. Fig. 10 shows an example of the latter category allowed to continue outgrowth for several days having clearly become the axon. Fig. 11 shows an example in which the initiated neurite was fixed and stained for dephospho-tau after 24 h of outgrowth. This neurite showed nearly complete compartmentation of the antigen to the axon, although not a gradient in this instance. In addition to neurites initiated de novo, there were five examples of axonal specification from experiments in which towing of an existing minor processes ceased before the experimental neurite became longer than sibling neurites. The short experimental neurites that became axons had 51 sibling minor processes among them at the end of towing, and only 2 of these showed any subsequent elongation. Clearly, specification of the experimental neurites was due to towing and not a random specification by chance. In the two cases of additional minor process outgrowth, the experimentally induced neurite persisted and so minor process elongation formed neurons with two apparent axons after several days of growth.

Discussion

Mechanical tension applied to hippocampal neurons can initiate and elongate processes into uniform-caliber neurites of considerable length. We and many others have observed that the neurites of hippocampal neurons at early times in culture fit into two clearly distinct categories, axon or minor process (Dotti et al., 1988; Craig and Banker, 1994). Thus, the neurites we elongated experimentally can only be axons or long minor processes. Four well-established markers indicate that our experimentally extended neurites differentiated into axons and were not simply long minor processes. These four indicators of axonal character are rapid growth cone advance rate, continued persistence and elongation similar to that of spontaneous axons, and axonal enrichment of dephospho-tau and of L1.

Rapid growth cone advance is one of the earliest and most frequently cited indicators of axonal specification in hippocampal neurons (Dotti et al., 1988; Craig and Banker, 1994). We found that if we applied intermittent force with a towing needle to mimic the periodic force that normally underlies axonal elongation by growth cones (Lamoureux et al., 1998), then the towed neurite developed a growth cone that routinely (re)attached to the substratum and advanced rapidly without the further aid of experimentally applied tension. We regard this unusually rapid growth cone activity to be a particularly convincing indicator of axonal character for several reasons. First, it provides evidence that tension stimulates the growth cone, the usual agent of neurite lengthening. That is, the rapid extension of one minor process to become an axon indicates that one growth cone has become more active than those of the sibling neurites, and neurite towing produced just the sort of substantial and routine stimulation of growth cone activity (Fig. 5) expected of axonal specification. Further, the towed neurite "volunteered" this growth cone activity insofar as the distal end of the neurite was initially otherwise engaged by attachment to the needle. Finally, no

Figure 11. **De novo initiation of short experimental neurite and subsequent spontaneous elongation and dephospho-tau compartmentation.** (A) A neuron with several fairly long minor process is stimulated by experimental application of tension to the cell margin (arrow). (B) After 3 h of towing, a neurite of 50 μm has been initiated, and the growth cone has moved beyond the needle (arrowhead) as in Fig. 5. This was taken as an opportunity to remove the needle (C). (D) 5 h after needle removal, the experimental neurite has elongated an additional 100 μm. (E) The next day, the experimental neurite elongated 100 μm further. (F) Shortly thereafter, the neuron was fixed and processed for dephospho-tau localization. This particular experimental neurite shows clear compartmentation of the antigen but not a gradient (see discussion of dephospho-tau localization in Results). This is also another instance (Fig. 5) in which one other minor processes elongated, but interestingly none of the spontaneous neurites show dephospho-tau localization. Bar, 20 μm.
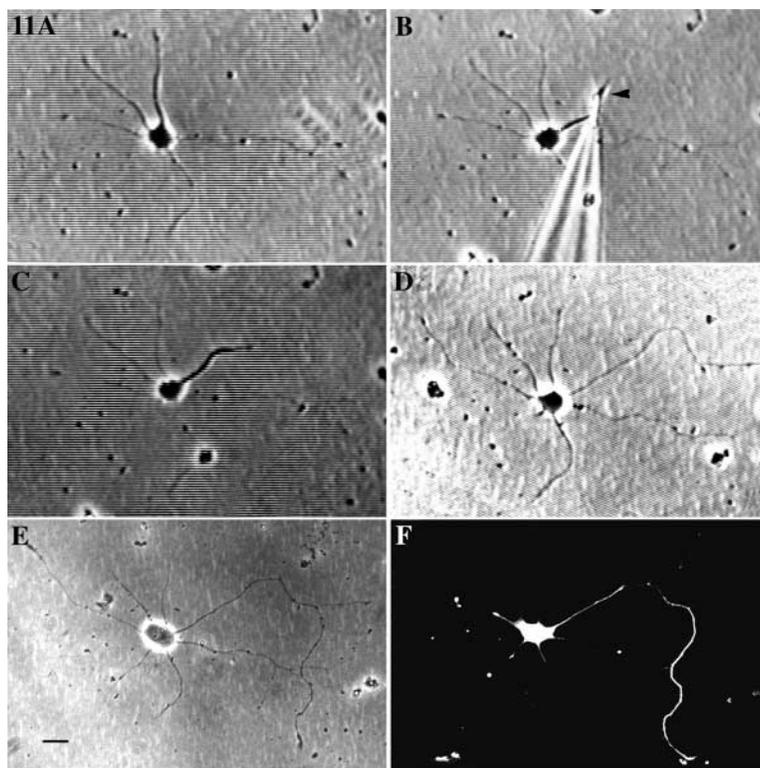
unmanipulated sibling minor process ever showed rapid outgrowth of this kind during the course of our observations. Growth cones of towed neurites were clearly behaving quite differently than those of the untowed siblings and in a way that is characteristic of early axonal specification.

In addition to rapid spontaneous growth cone activity, the difference between an axon and a minor process is also partly defined by the persistence of growth by an axon, leading to its substantial increase in length over time. In our experiments, when the towing needle was removed successfully and without damaging the neurite the extent and persistence of outgrowth by experimentally induced neurites were at least as good as that of the axons of early stage 3 axons that were spontaneously specified. Among experimental neurites, 80% remained extended or grew, whereas among spontaneous axons 67% of initial axons remained extended or grew. Among experimental neurites, ∼30% doubled their length after the experimental intervention (Figs. 2, 6, 7, and 11), whereas 15% of spontaneous axons doubled their length over the next day or two and, surprisingly, another 15% grew substantially by elongating a second axon. In this latter regard, Ruthel and Hollenbeck (2000) found that 4% of individually identified stage 2 (not early stage 3 as in our observations) spontaneously grew two axons in only 14 h of observation. In addition to elongation characteristics, the frequency of "failure" in axonal specification was also similar among experimental and spontaneous axons at early times of specification. Some 20% of experimentally induced neurites failed to persist, whereas 33% of spontaneous stage 3 neurons showed regression of an initially specified axon. Thus, at early times of apparent axonal specification both experimentally induced neurites and spontaneous axons showed very similar extents and persistence of growth.

Dephospho-tau and L1 are among the earliest molecular markers for axonal specification (Van den Pol and Kim, 1993; Mandell and Banker, 1996; Ruthel and Hollenbeck, 2000). Both showed compartmentation to experimental neurites at the end of the towing period with uniform distribution for dephospho-tau or uniformly punctate distribution for L1 (Fig. 9). Because dephospho-tau compartmentation and gradient have been reported to take additional days of outgrowth after axonal specification, we analyzed dephospho-tau compartmentation in several experimental cells allowed to incubate after towing. Most experimental neurites that were incubated after towing showed the distal to proximal gradient of dephospho-tau characteristic of hippocampal axons. This gradient of dephospho-tau in experimental neurites was similar in appearance to that of spontaneously specified axons, and both showed good reproducibility of the phenomena. Thus, the identity of the experimental neurites as axons is further supported by immunostain for the molecular marker dephospho-tau.

Attainment of a critical length by one minor process, and therefore the breaking of symmetry, has been hypothesized to be the point at which an axon is specified (Goslin and Banker, 1989). As our studies have shown, tension and axonal elongation are very closely related. However, because growth cone advance and neurite lengthening can only occur as a result of a force, from a physical point of view force necessarily precedes lengthening. We were able to experimentally separate these two closely related parameters by showing that tension could induce axonal fate even when we ceased towing while the experimental neurite was shorter than its siblings. Our best success was achieved by initiating neurites de novo where, once again, alternation of tension and relaxation stimulated growth cone activity. These exper-

imentally initiated, short neurites also developed into axons as shown by continued outgrowth and compartmentation of dephospho-tau (Figs. 10 and 11).

Another common theme in models of axon specification is the existence of an unidentified inhibitor that prevents more than one neurite from becoming an axon (Craig and Banker, 1994; Andersen and Bi, 2000; Bradke and Dotti, 2000). This is meant to explain the finding that hippocampal neurons normally develop only one axon, and when the axon is transected to the length of its siblings, each minor process (including the transected axon) has an equal chance of becoming the new axon (Dotti and Banker, 1987; Goslin and Banker, 1989). Surprisingly, tension-mediated elongation of minor processes and neurite initiation de novo were no more difficult to achieve with stage 3 neurons, which already possessed an axon, than it was with stage 2 neurons. In fact, experimental neurite behavior was indistinguishable among stage 2 and stage 3 neurons in 1–2-d-old cultures. That is, presence of an axon did not affect ease of needle attachment, forces required for elongation, tendency for detachment while under tow, likelihood of spontaneous growth cone advance, subsequent longer term growth, or high levels of dephospho-tau immunofluorescence. (Rather, the only instances in which experimental elongations of minor processes were problematic were in stage 2 neurons from 3–4-d-old cultures. We presume this reflects a general incapacity for neurite elongation in these neurons. That is, if the cell had not extended an axon by 3–4 d, then it appeared to be incapable of supporting substantial elongation either spontaneously or experimentally.) However the formation of multiple axons might normally be inhibited, our results on stage 3 neurons indicate that such inhibition is easily overcome by the stimulus of mechanical tension. In this regard, we found among individually identified neurons in early, spontaneous stage 3 that formation of a second axon and of axonal respecification is more frequent than we expected. As noted earlier, Ruthel and Hollenbeck (2000) reported that 4% of individually identified stage 2 neurons developed two axons during 14 h of observation. Also, Bradke and Dotti (2000) reported that even well-differentiated dendrites of hippocampal neurons could become axons and that actin depolymerization could induce a high frequency of multiple axons. The presumptive inhibitory influence of an axon on the growth of other neurites may be less robust than once thought.

The results of this study establish that mechanical tension is a proximate stimulus to break symmetry in a neuron and specify which process becomes the axon. In the uniform environment of a culture dish, this occurs as a stochastic choice among minor processes as first suggested by Craig and Banker (1994). That is, during the growth and retraction of minor processes that occurs in stage 2 neurons the first growth cone that happens to exert enough tension to begin rapid and sustained elongation becomes the axon. In the animal, the outcome of this stochastic "tug of war" (Bradke and Dotti, 2000) is undoubtedly affected by the environment surrounding the cell. The growth cones of the minor processes probe their environment for chemical signals that stimulate a particular growth cone to exert more force and thus lead to axon specification (Brittis and Silver, 1995; Esch et al., 1999). Regardless of whether or not environ-

mental cues are present, there must be proximate chemical stimuli that underlie the generation of mechanical tension. Effective tension is ultimately dependent on a variety of intracellular conditions, presumably including actin dynamics and myosin function. For example, recent studies suggest that axonal specification and growth rate is sensitively dependent on expression of proteins expected to increase actin turnover (Meberg and Bamburg, 2000; Kunda et al., 2001). In our view, the current study complements biochemical investigations by showing that there is a clear physical basis for the development of a minor process into an axon, which in turn is dependent on biochemical dynamics.

Materials and methods

Culture of hippocampal neurons
Hippocampal neurons were cultured from embryonic rats (E18 or E19) based on the method of Goslin and Banker (1991) with several modifications. After trituration of cells, cells were frozen in commercial cell culture freezing medium (11101–011; Life Technologies, GIBCO-BRL) in aliquots that each represented the cells obtained from a single hippocampus. Aliquots were stored in liquid N_2 until use. As reported previously, hippocampal neurons cultured after cryopreservation were indistinguishable from fresh cultures (Mattson and Kater, 1988; Ruthel and Hollenbeck, 2000). After thawing, cells were plated onto poly lysine–treated tissue culture dishes and initially cultured in a 5% CO_2 atmosphere for 2–4 h in glia-conditioned (Goslin and Banker, 1991) MEM medium (61100–053; GIBCO-BRL) containing 2 mM glutamine, 0.6% glucose, 10% horse serum, 100 U/ml of penicillin, and 100 μg/ml streptomycin. After this initial culture period in bicarbonate-buffered medium, cells were transferred to glia-conditioned CO_2-independent medium (18045–088; Life Technologies) containing 4 mM glutamine, 1 mM pyruvate, 0.6% glucose, 10% horse serum, 100 U/ml of penicillin, 100 μg/ml streptomycin, and several defined growth supplements, including insulin, transferrin, estradiol, etc., as previously detailed for the culture of chick brain neurons (Heidemann et al., 2001). These additions dramatically improve the frequency of axonal development in embryonic chick brain neurons, and we found that they also promote the development of rat hippocampal neurons.

Application of experimental tension to minor processes
Force was applied to hippocampal neurons with calibrated glass needles using a method that has been described in detail elsewhere (Heidemann et al., 1999). Briefly, two needles were mounted in a micromanipulator; one needle was calibrated for its bending constant and used as a pulling needle applied to the cell, whereas the other needle was used as an unloaded reference for bending of the towing needle and for assessing movements of the system imposed through the micromanipulator. The bending constants of the pulling needles were between 3–9 μdyne (30–90 pN)/μm, and needles were pretreated with poly lysine (0.1% in PBS) and subsequently with concanavalin A (10 mg/ml in PBS) to aid attachment of the cell to the needle. All applications of force were recorded by videotape at 48–120× time lapse for subsequent analysis of neurite length and needle bending (a measure of force magnitude).

To determine the relationship between applied tension and elongation rate, a calibrated needle was attached to the growth cone of a spontaneous neurite, and the neurite was pulled in 60–100-min "steps" of constant force (Zheng et al., 1991). That is, a tension magnitude was chosen, beginning at 30–50 μdyne, and this tension was held constant for >1 h by moving the micromanipulator to maintain the appropriate deflection of the calibrated needle. Subsequently, the same technique was used to apply 60–100-min periods of higher tension to the neurite, each level typically 20–50 μdyne higher than the previous value. Neurite elongation was measured from the videotape record of the experiment. For each period of constant force, a rate of elongation was calculated from the change in neurite length divided by the period of time that force was applied.

After most towing experiments, attempts were made to manipulate the distal end of the neurite off the needle and back onto the surface of the dish for observation of independent growth by the towed neurite or for immunofluorescent analysis. Needle removal was more difficult for hippocampal neurons than for other cultured neurons with which we have worked owing to the much greater tendency of hippocampal neurites to break and for the cell body to detach in response to the forces needed to

detach the neurite. Large numbers of neurons successfully manipulated were lost as a result of attempted needle removal. Cultures that were incubated after experimental manipulations were maintained in glia-conditioned CO_2-independent medium containing horse serum and supplements as described above or in this same medium with an overlay of rat glia on a coverslip (Goslin and Banker, 1991).

Immunofluorescent analyses

Experimental cells successfully removed from the needle were marked with a circle made on the bottom of the dish with a diamond-tipped microscope "objective." Immunofluorescent staining for dephospho-tau was performed by a method similar to that of Ruthel and Hollenbeck (2000). Medium was carefully removed, and the cells were fixed in a freshly prepared solution of 4% paraformaldehyde and 4% sucrose for 20 min. After two rinses in PBS, the cells were permeabilized by a 10-min incubation in 0.2% Triton X-100 in PBS. Cell were then incubated in 3% goat serum and 2% BSA in PBS for 30 min and then in an antibody specific for dephospho-tau (tau-1, 1:200; Boehringer) for 2 h at 37°C. After three rinses with PBS, cells were incubated in Alexa-488–conjugated secondary antibody (1:1,000; Molecular Probes) for 1 h at room temperature. After three more rinses in PBS, coverslips were mounted over the appropriate region of the tissue culture dish with Aqua Polymount (Polysciences Inc.). Immunofluorescent staining for L1 was performed by a similar procedure except that the permeabilization step was omitted and the primary anti-L1 antibody was a gift from Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). This was diluted 1:10,000 in 2% horse serum in PBS and incubated with the sample overnight in the refrigerator as recommended by Dr. Lemmon.

Immunofluorescent staining for microtubules was performed by a method similar to that of Thompson et al. (1984). Cells were lysed and stabilized for 3 min at 37° in 0.5% Triton X-100 in the microtubule-stabilizing buffer described by Thompson et al. (1984). The cells were then fixed for 20 min with freshly prepared 4% paraformaldehyde in microtubule-stabilizing buffer. After rinsing with PBS, cells were blocked with 3% goat serum and 2% BSA in PBS for 30 min. Cells were then reacted with a primary antibody against β-tubulin (1111876; Boehringer) for 1 h and washed three times with PBS. The fluorescent secondary antibody was goat anti–mouse IgG AlexaFluor 488 (A11001; Molecular Probes). After rinsing three times in PBS, the cells were mounted as above.

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