Motor proteins regulate force interactions between microtubules and microfilaments in the axon

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It has long been known that microtubule depletion causes axons to retract in a microfilament-dependent manner, although it was not known whether these effects are the result of motor-generated forces on these cytoskeletal elements. Here we show that inhibition of the motor activity of cytoplasmic dynein causes the axon to retract in the presence of microtubules. This response is obliterated if microfilaments are depleted or if myosin motors are inhibited. We conclude that axonal retraction results from myosin-mediated forces on the microfilament array, and that these forces are counterbalanced or attenuated by dynein-mediated forces between the microfilament and microtubule arrays.

Axons grow over potentially long distances to reach their target tissues during the development of the nervous system. Axon formation involves periods of retraction and of elongation, both of which are essential for appropriate navigation to the target[1]. Within the axoplasm are dense arrays of cytoskeletal filaments; during axonal elongation and retraction, these components, particularly microtubules and microfilaments, undergo rapid changes[2]. Microtubules are hollow cylindrical filaments that are relatively stiff and are therefore thought to bear compressive forces. They are organized into a paraxial bundle that traverses the length of the axon[3–5]. Some microfilaments are also arranged paraxially within the axon, but a significant proportion are organized into a cortical mesh[6–9]. The microtubule and microfilament systems of the axon are integrated with one another to facilitate regulation of axonal events such as elongation or retraction[10–15]. Given that microtubule depletion causes axons to retract, and that this retraction can be rescued by microfilament depletion[16,17], it has been suggested that microtubules may provide the structural support needed to bear the tension imposed by the microfilament system. In this model, the degree of tension determines whether an axon grows or retracts; elongation and retraction of the axon would therefore be regulated by shifts in the relative levels of microtubules and microfilaments[18].

Many workers have found this model attractive because it is based on classic architectural principles used to build a variety of structures, such as geodesic domes. However, living cells are highly dynamic and, as such, are more comparable to a machine than to a static architectural structure. It therefore seems more reasonable to suggest that the integration of the microtubule and microfilament systems may be mediated by dynamic forces. Cytoplasm is rich in a class of enzymes known as molecular motor proteins, which hydrolyse ATP and use the energy thereby released to generate forces relative to microtubules or microfilaments. For several years, studies of motor proteins focused on their function in transporting organelles along the surfaces of cytoskeletal elements, but it is now evident that motor proteins also transport and organize the cytoskeletal elements themselves. For example, the formation and function of the mitotic spindle are dependent on a host of motor-mediated forces[19,20].

It seems likely that the tension exerted by the microfilament system of the axon is generated by one or more members of the myosin family, although this has never been tested directly. With respect to the identity of the motor that may generate forces between the microtubule and microfilament systems, cytoplasmic dynein is an attractive candidate. Although this motor was previously thought to function mainly in retrograde vesicle transport, there is now a great deal of evidence indicating that it may also generate forces between microtubules and microfilaments in a variety of cell types. These forces are thought to position microtubules and centrosomes during interphase[21–23], drive apart the duplicated centrosomes during prophase and anaphase[24–27], and transport microtubules from the centrosome into the developing axons of postmitotic neurons[28]. Here we seek to test the hypothesis that the axonal microfilament system is subject to strong contractile forces generated by myosin, and that these forces are counterbalanced by forces between the microfilament and microtubule arrays generated by cytoplasmic dynein. Our results show that retraction phenomena, similar to those caused by depletion of cytoskeletal polymers, can also be observed when polymers are left intact and motor-mediated forces are inhibited.

Results

Our hypothesis predicts that both elongation and retraction of the axon would depend on the action of motor-mediated forces on cytoskeletal elements. Unfortunately, we were not able to test this prediction with respect to axonal elongation, because the motor proteins involved in our proposed model are known to have other crucial functions in this process. For example, myosins are important for transporting vesicular elements to the growing tip of the axon[29], and cytoplasmic dynein is important for the retrograde transport of membranous vesicles, many of which contain important growth factors[30]. Thus the effect of inhibition of these motors on axonal elongation would be difficult to interpret. We therefore focused on axonal retraction, as it seems unlikely that a rapid retraction response could be produced by suppressing the membrane-transport functions of these motors, at least over relatively short time periods. To speed up the retraction response, we grew chick sensory neurons on untreated glass coverslips, which constitute a very poorly adhesive substrate. Under these conditions, axons are typically adhered only at their growth cones, and are only loosely associated with the substrate along their lengths[31].

Effects on the axon of depletion of microtubules and microfilaments. The results of previous pharmacological studies indicate that axonal retraction may be counterbalanced by some feature of

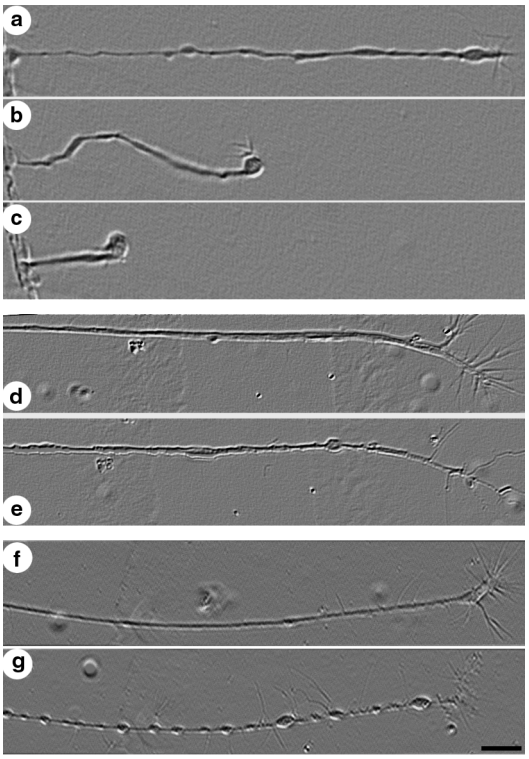

Figure 1 **Effect of nocodazole, nocodazole and latrunculin, or nocodazole and NEM-modified-S1 on axonal retraction.** Time-lapse images of axons subjected to the indicated treatments. **a**–**c**, Axon treated with nocodazole. Images were obtained before drug application (**a**), and 15 min (**b**) and 30 min (**c**) after. **d**, **e**, Axon treated with nocodazole and latrunculin. Images were obtained before drug application (**d**) and 30 min after (**e**). **f**, **g**, Axon treated with nocodazole and injected with NEM-modified S1. Images were obtained before experimental treatment (**f**) and 30 min after (**g**). Axons frequently, but not always, showed substantial beading (as in **g**) along their lengths in response to nocodazole treatment. Scale bar represents 15 μm.

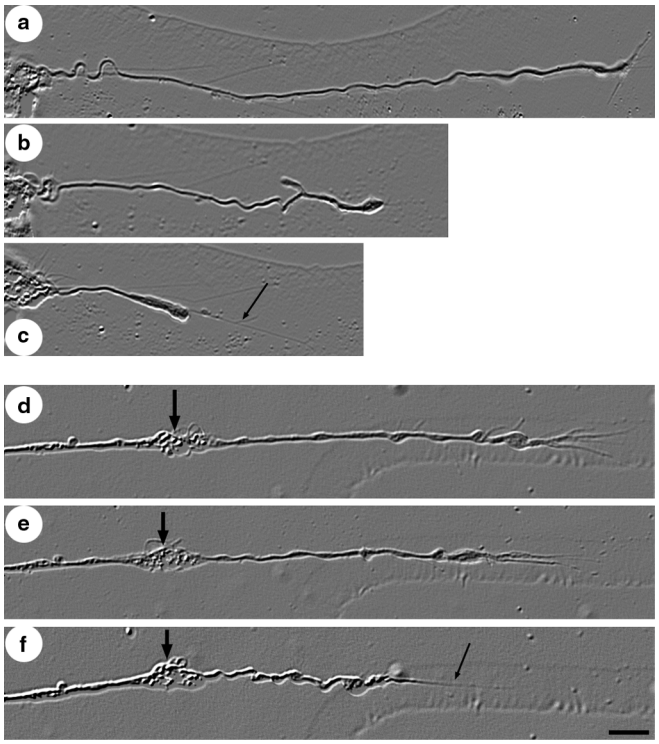

Figure 2 **Effect of recombinant dynamitin on axonal retraction.** Time-lapse images of axons injected with dynamitin. **a**–**c**, Images were obtained shortly after injection (**a**), and 15 min (**b**) and 30 min (**c**) after. No axonal beading was observed, but axons showed marked sinusoidal bending during retraction. **d**–**f**, An axon attached to another cell part way along its length (thick arrows). Images were obtained shortly after injection (**d**), and 15 min (**e**) and 30 min (**f**) after. Axons retracted disto-proximally. Sinusoidal bending mainly occurred distal to the point of attachment to the other cell over the first 30 min of retraction. Thin arrows show 'strands' trailing behind the distal tip of the retracting axon. Scale bar represents 15 μm.

the microtubule array[16,17]. To generate an appropriate benchmark with which to compare the results of motor-inhibition studies, we revisited these earlier studies, using our experimental system. We used nocodazole to depolymerize microtubules because it is a highly specific anti-microtubule drug, the effects of which on the microtubule array of these neurons have previously been investigated. Treatment with nocodazole for 15–30 min depolymerizes a substantial portion of the microtubule mass along the axon shaft and nearly all of the microtubule mass within the more distal region of the axon[32,33]. We found that 10 μg ml^{-1} nocodazole caused axons to begin retracting within a few minutes of application; dramatic retraction was observed in all cases (n=37). Retraction continued at an average rate of 2.8±0.9 μm min^{-1} (Fig. 1a–c). In many, but not all, cases, axons developed prominent 'beads' along their lengths, a classic morphological symptom of microtubule depolymerization. Within an hour of drug treatment, 35 of the 37 axons (all of which were originally 75–100 μm in length) had completely retracted into the cell body.

To investigate the importance of microfilaments in these retractions, we pretreated cultures with the anti-actin drug latrunculin (0.2 μg ml^{-1}) for 15 min before adding nocodazole. A few experiments were also carried out using another anti-actin drug, cytochalasin D (10 μg ml^{-1}), which produced results that were indistinguishable from those for latrunculin. Latrunculin causes microfilament disassembly by binding to unassembled actin subunits, thereby removing them from the polymerization/depolymerization cycle[34], whereas cytochalasin D disrupts microfilaments by binding to the filaments themselves[12]. The anti-actin drugs, by themselves, caused no noticeable retraction of the axon, although axonal edges became somewhat 'wavy' in appearance in some cases. Consistent with results obtained from other neuronal systems[16,17], the anti-actin drugs almost completely abolished the retraction induced by nocodazole in all axons studied (n=23; Fig. 1d–e). When the anti-actin drugs were rinsed from nocodazole-treated cultures, retraction rapidly ensued in all cases (n=6; data not shown). These results support previous findings that axonal retraction requires microfilaments, and that this microfilament-dependent retraction is counterbalanced by microtubules.

Effects of inhibition of myosin activity on nocodazole-induced axonal retraction. The finding that axonal retraction is an energy-dependent process[16,31,35] is consistent with the idea that it involves contractile forces generated by one or more of the myosin motors. To investigate this possibility, we sought a way to effectively and specifically inhibit myosin activity while leaving microfilaments intact. The pharmacological agent BDM has previously been used to inhibit myosin[36], but concerns have been expressed regarding its specificity. We found that we could not use BDM for our analyses because it caused cultured chick sensory neurons to shrivel and rapidly detach from the substrate, presumably as a result of nonspecific toxicity. A more useful approach for inhibiting myosin activity was to microinject cells with excess amounts of a version of the myosin motor (S1) domain that had been chemically modified with *N*-

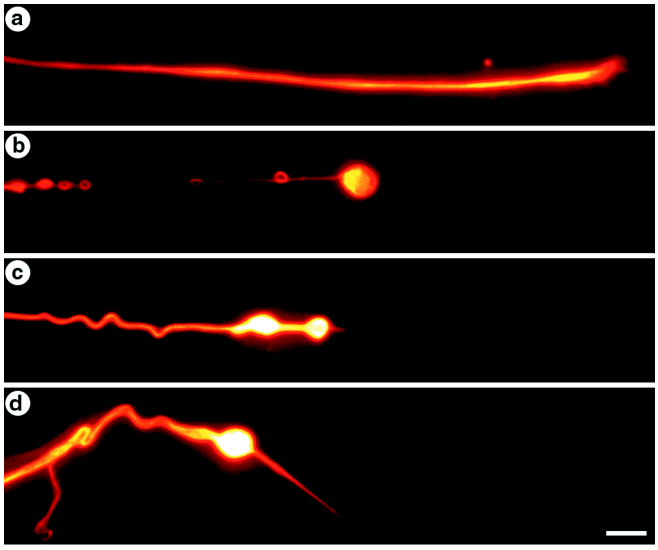

Figure 3 **Quantitative immunofluorescence analysis of microtubule concentration and distribution in retracting axons.** After experimental manipulations, cells were fixed and prepared using a general antibody against β-tubulin. Red represents the lowest fluorescence intensity and white represents the highest. **a**, Control axon. **b**, Axon treated with nocodazole for 30 min. **c**, **d**, Axons 30 min after injection of neurons with dynamitin. All axons except the control retracted by at least a third of their original lengths. Note that the nocodazole-treated axon shows a clear diminution in polymer concentrations, whereas axons of dynamitin-injected neurons show significantly higher concentrations of polymer. Total fluorescence intensities in **a**, **c** and **d** are roughly equal, showing that little or no microtubule depolymerization occurs during retraction. The high levels of fluorescence in distal regions of retracting axons indicate that microtubules may retreat disto-proximally from the axon tip during retraction. **d** shows that the trailing strands often left during dynamitin-induced retraction contain microtubules. Scale bar represents 18 μm.

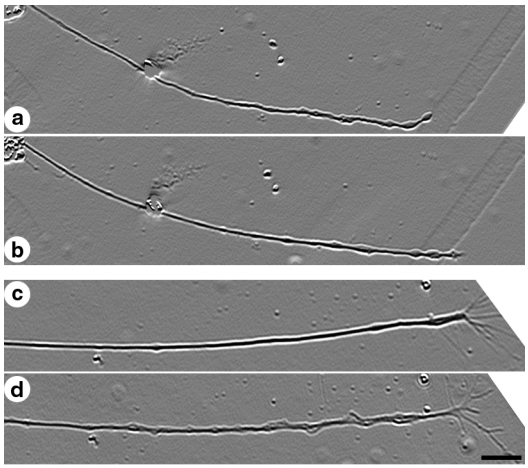

Figure 4 **Effects on axons of dynamitin in combination with latrunculin or with NEM-modified S1.** Time-lapse images of axons subjected to the indicated treatments. **a**, **b**, Axon pretreated with latrunculin for 15 min and injected with recombinant dynamitin. Images were obtained before experimental manipulation (**a**) and 30 min after injection (**b**). **c**, **d**, Axon co-injected with dynamitin and NEM-modified S1. Images were obtained before injection (**c**) and 30 min after (**d**). No retraction was observed with either treatment. Scale bar represents 15 μm.

ethylmaleimide (NEM). This 'NEM-modified S1' interacts with microfilaments and produces rigor complexes that inhibit the interaction of endogenous myosins with microfilaments[36–38]. The myosin 'heads' are purified from skeletal muscle and are exclusively of the myosin-II type. However, because they interact directly with the microfilaments themselves, they presumably inhibit force generation by any of the myosin-family proteins. The capacity of NEM-modified S1 to bind to microfilaments and to form rigor complexes has been confirmed visually, using electron microscopy, and functionally, in studies showing that it inhibits myosin-dependent events such as the reorganization of microfilaments during cytokinesis[37,38] and microfilament movements within neuronal growth cones[36].

Injection of NEM-modified S1 (30–100 mg ml^{-1}) into cultured sensory neurons produced results similar to those obtained from latrunculin treatment — no axons showed retraction ($n = 19$), although axonal edges sometimes became slightly wavy in appearance. When neurons were microinjected with NEM-modified S1 before addition of nocodazole, the observed responses were similar to those caused by treatment with latrunculin or cytochalasin D. In all cases, axons showed very little or no retraction ($n = 24$; Fig. 1f, g), but we commonly observed substantial axonal beading. Retraction was not inhibited if NEM-modified S1 was denatured by boiling before injection. These results indicate that retraction is dependent not only upon microfilaments, but also upon myosin-mediated forces.

Effects of inhibition of the activity of cytoplasmic dynein on the axon. It has been argued that microfilament-dependent retraction of axons is counterbalanced by the presence of microtubules as structural elements[18]. We tested the alternative hypothesis that it is not the mere presence of microtubules that counterbalances acto-myosin-dependent retraction, but dynein-mediated forces. To specifically and rapidly inhibit dynein activity, we microinjected recombinant dynamitin protein into neurons. Dynamitin is a component of the dynactin complex that, when present at abnormally high levels, causes the complex to dissociate. Dynactin is required for all known functions of cytoplasmic dynein, and hence a dissociation of the complex results in an immediate cessation of dynein activity,[39,40]. Introduction of roughly 4 pl of recombinant dynamitin at 3 mg ml^{-1} (previously shown to be effective in inhibiting dynein function in cultured neurons[28]) caused axons to start retracting within a few minutes of injection in 35 out of 38 cases. Retractions induced by dynamitin were slower (1.9±0.5 μm min^{-1}) than nocodazole-induced retractions, and were not accompanied by axonal bead formation, but were instead consistently accompanied by the formation of sinusoidal bends along the length of the axon (Fig. 2a–c). In axons that were adhered, part way along their lengths, to another cell, sinusoidal bending occurred first at a point distal to the attachment point, and thereafter between the cell body and the attachment point (Fig. 2d–f). These bends were very similar to those observed in axons that retract as a result of transection[31,32]. Observations from electron microscopy indicate that these bends may result from the presence of long microtubules, which must be gradually compressed backwards during retraction[35]. There was no apparent lifting or loosening of the axon from the substrate during retraction. In almost all cases of dynamitin-induced retraction, we observed thin strands that trailed behind the retracting axon (Fig. 2c, f). Injection of dynamitin that had been denatured by boiling did not cause retraction (data not shown).

The simplest interpretation of these results is that the axon would normally retract and microtubules would normally retreat backwards during retraction, if not for dynein-mediated forces on the microtubule array. However, this assumes that dynamitin injection did not somehow induce microtubule disassembly, which would also cause the axon to retract. To confirm this, we used quantitative immunofluorescence microscopy (Fig. 3) to measure microtubule concentrations in axons that were retracting, as a result either of nocodazole treatment or of dynamitin injection. We

Table 1 Summary of data from axonal retraction assays

Treatment	Without nocodazole or dynamitin	Nocodazole	Dynamitin
Without latrunculin or NEM-modified S1	No retraction	Retraction ($2.18\pm0.9\,\mu m\,min^{-1}$; $n=37/37$)	Retraction ($1.9\pm0.5\,\mu m\,min^{-1}$; $n=35/38$)
Latrunculin	No retraction ($n=50/50$)	No retraction ($n=23/23$)	No retraction ($n=17/17$)
NEM-modified S1	No retraction ($n=19/19$)	No retraction ($n=24/24$)	No retraction ($n=17/17$)

'No retraction' was defined as shortening of the axon by <5 μm

selected axons of similar length (~100 μm; $n=5$), and examined them after 30 min of treatment. The 'strands' left during dynamitin-induced retraction (Fig. 3d) contained microtubules, indicating that some microtubules may recede more slowly than others. The total fluorescence intensities in the axons of control, nocodazole-treated and dynamitin-injected neurons were 1,934±417, 661±271 and 1,959±498 arbitrary fluoescence units, respectively. Thus, substantial microtubule disassembly was caused by nocodazole treatment but not by injection of dynamitin. Retracting dynamitin-injected axons were particularly bright in their distal regions, which is consistent with a disto-proximal retreat of microtubules during retraction.

Effects of microfilament depletion or myosin inhibition on axonal retraction. Pretreatment with latrunculin almost completely abolished dynamitin-induced retraction in all cells ($n=17$; Fig. 4a, b), as did co-injection of NEM-modified S1 with dynamitin ($n=17$; Fig. 4c, d). In cells pretreated with latrunculin, retraction ensued after rinsing out the drug (data not shown). These observations confirm that dynamitin-induced retractions are indeed due to actomyosin-derived forces. We therefore conclude that dynein-mediated forces between the microtubule and microfilament arrays counterbalance actomyosin-based retraction. Table 1 summarizes our data on axonal retraction under the indicated experimental regimes.

Discussion

It is now clear that the establishment and regulation of cellular cytoskeletal arrays cannot be explained solely in terms of static architectural principles. It was previously proposed that microtubule and microfilament arrays are generated, organized and regulated by the assembly properties of cytoskeletal polymers and their related structural proteins that stabilize and bundle them. However, this view does not satisfactorily explain the formation and functioning of complex cytoskeletal arrays such as the mitotic spindle or the microtubule and microfilament systems within the axons and dendrites of postmitotic neurons. Evidence now indicates that complex cytoskeletal arrays may be configured by forces generated on microtubules and microfilaments by molecular motor proteins. These motors recognize the polarity of the filaments as well as other cytoplasmic structures with which the filaments are juxtaposed. The best studied example of motors acting in this manner is provided by the mitotic spindle, which requires motor-driven forces for its formation as well as its transitions from one stage of cell division to another[19,20].

These new findings challenge the older idea that the cytoskeleton is a 'tensegrity structure' composed of two sets of structural elements, one under tension and the other under compression. Many of the fundamental principles underlying this model are probably correct, but it is not sufficient to explain fully how cells organize and regulate their cytoskeletal arrays[41]. Nevertheless, the tensegrity model provided an attractive explanation of the relationship between microtubules and microfilaments within the axon. Microtubules were thought to be stiff elements that are able to bear the compressive forces exerted by microfilaments, which somehow generate tension that must also be borne[17,18,42]. However, no explanation is provided of the initial establishment of this relationship or the source of energy needed for generation of tension. More recently, it has been shown, in other cell types, that abrupt depolymerization of microtubules initiates a biochemical cascade that can potentially alter actin dynamics or enhance phosphorylation of the myosin light chain[43–47]. These observations undoubtedly reflect mechanisms that function in developing neurons as well as other cells, but they do not address possible motor-mediated interactions that may link the microtubule and microfilament systems more directly. A complete model would incorporate, as a fundamental principle, the generation of forces between microtubules and microfilaments by motor proteins, especially as motor-mediated forces are known to organize other kinds of cytoskeletal arrays, such as the mitotic spindle.

We have tested a new model for the integration of the microtubule and microfilament arrays within axons, which involves forces generated by myosin and cytoplasmic dynein. In this model, myosin generates forces on the microfilament array that are responsible for generating the 'tension' within the axon. As a result of the meshwork configuration of cortical microfilaments, these myosin-generated forces would be contractile, and hence, if not sufficiently antagonized, would cause the axon to retract. These actomyosin forces are counterbalanced or attenuated in some way by forces, generated by cytoplasmic dynein, between the microfilament and microtubule arrays. Our model is attractive because it can explain the relationship between microtubules and microfilaments in the axon more comprehensively than can a purely structural model, and because it is consistent with evidence from a variety of cell types indicating that cytoplasmic dynein may generate forces between microtubules and microfilaments[21–27].

Our results show that inactivation of cytoplasmic dynein does not induce any significant depolymerization of microtubules, but still causes the axon to retract. Similarly, inactivation of myosin proteins produces an effect comparable to that of microfilament depletion, in which axonal retraction is rescued. Thus, when cytoplasmic dynein is inactivated, the microtubule system remains assembled but it is incapable of counterbalancing the strong contractile forces generated by the actomyosin system. The precise mechanism of integration of these two systems by dynein-generated forces remains unclear, and could potentially involve several factors. Given that the principal function of a motor protein is to generate forces, and that such forces can cause microtubules to move, one possible explanation for our results relates to the proposed function of cytoplasmic dynein in microtubule transport. It has been suggested that cytoplasmic dynein may drive microtubules down the axon by generating forces on the microfilament array[28,48,49]. It seems reasonable that these forces could create a 'drag' on the microfilament system that would attenuate its contractility.

We conclude that it is not the mere presence of these filaments that accounts for their importance in regulating axonal elongation and retraction, but rather that the crucial factor is the motor-mediated forces that act upon them. Inactivation of kinesins in fibroblasts can produce very similar results to those of microtubule depletion[50], indicating that this model may be broadly applicable to different cell types and to other motor proteins. Motor-generated forces may be a principal means by which the cytoskeletal arrays of living cells are integrated with one another to organize cellular morphology. With regard to the development of the nervous system, the tight regulation of motor-mediated forces offers a powerful mechanism by which bouts of axonal elongation and retraction could be modulated in response to a variety of external cues. It seems sensible to imagine that axons undergo elongation and retraction by reconfiguring their cytoskeletal polymers (sliding them backwards and forwards), rather than by constantly assembling and disassembling their microtubule and microfilament arrays. □

Methods

Cell culture.

Cultures of chick sensory neurons were prepared from the dorsal root ganglia of E11 chicks using a modification of previously described methods[31,32]. Ganglia were cleaned, treated with 0.25 mg ml^{-1} trypsin and 0.25 mg ml^{-1} collagenase for 15 min, and then triturated with a pasteur pipette into a single-cell dispersion. Neurons were then plated onto 'special dishes' that were prepared by attaching an acid-washed glass coverslip (Bellco Glass, Vineland, NJ) to the bottom of a 35-mm plastic petri dish, into which a 10-mm hole had been drilled. Coverslips had been photo-etched with a pattern of boxes to assist in relocation of individual cells and to provide reference marks to assess the extent of axonal retractions. Neurons were plated in a modified L15-based medium containing Leibovitz' L15 (Sigma) supplemented with 0.6% glucose, 2 mM L-glutamine, 0.6% methyl cellulose, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 100 µg ml^{-1} nerve growth factor (Upstate Biotechnology, Lake Placid, NY). This medium promoted robust axonal outgrowth, and maintained pH in normal air.

Experimental treatments.

Nocodazole and cytochalasin D were from Sigma. Latrunculin A was from Molecular Probes. Stock solutions were prepared in dimethylsulphoxide at 1,000 times the final concentration used in the experiments. For introduction into the cultures, an equal quantity of medium containing twice the final concentration of the drug was added. This procedure minimized any mechanical disturbances that might otherwise have arisen from a requirement for agitation of the dishes to distribute the drug. Recombinant dynamitin was prepared as described[40] and microinjected into neurons in a standard microinjection buffer at a concentration of 3 mg ml^{-1} and a volume of roughly 4 pl. NEM-modified S1 was prepared from rabbit muscle tissue as described[36,38] and injected at a concentration of 30–100 mg ml^{-1} and a volume of roughly 4 pl. As negative controls, dynamitin and NEM-modified S1 were microinjected after denaturation by boiling. Experiments were carried out on the heated-stage of an Axiovert microscope (Zeiss). Images were obtained using differential-interference contrast (DIC) optics and were archived with a charge-coupled device (CCD; Photometrics PXL, Tucson, AZ). Unevenness in the brightness of individual DIC images was corrected using the 'emboss' function of Adobe Photoshop (Adobe Systems, San Jose, CA).

Fluorescence microscopy.

For observation of microtubules, cells were simultaneously fixed and extracted for 5 min in a solution containing PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 2 mM $MgCl_2$, pH 6.9), 4% formaldehyde, 0.15% glutaraldehyde and 0.2% Triton X-100. It was necessary to simultaneously fix and extract (rather than extract and then fix) because retracting axons were so poorly adhered to the substrate that they consistently detached if extraction was attempted before fixation. The marked diminution in fluorescence intensity in nocodazole-treated cultures allowed confidence that the simultaneous fixation and extraction procedure resulted in rapid enough permeabilization to release most or all of the free tubulin, thus permitting vizualization of microtubules. After fixation, cultures were rinsed and exposed for 30 min to a Cy3-conjugated monoclonal antibody against β-tubulin (Sigma), used at 1:100. After rinsing, cultures were mounted in a medium that reduced photobleaching (100 mg ml^{-1} DABCO and 1 mg ml^{-1} phosphorylated phenylenediamine in 90% glycerol and 10% PBS). Individual cells of interest were relocated, and images were acquired with a cooled CCD (Photometrics PXL), using Metamorph software (Universal Imaging, West Chester, PA). Identical settings and exposures were used for acquisition of all images, so that fluorescence intensities of samples could be compared quantitatively.

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