Kinesin-5 Is Essential for Growth-Cone Turning

Vidy C. Nadar,1 Andrea Ketschek,1 Kenneth A. Myers,1 Gianluca Gallo,1,2 and Peter W. Baas1,2,*
1Department of Neurobiology and Anatomy
Drexel University College of Medicine
2900 Queen Lane
Philadelphia, PA 19129
USA

Summary

Inhibition of kinesin-5, a mitotic motor protein also expressed in neurons [1], causes axons to grow faster as a result of alterations in the forces on microtubules (MTs) in the axonal shaft [2–4]. Here, we investigate whether kinesin-5 plays a role in growth-cone guidance. Growth-cone turning requires that MTs in the central (C-) domain enter the peripheral (P-) domain in the direction of the turn. We found that inhibition of kinesin-5 in cultured neurons prevents MTs from polarizing within growth cones and causes them to grow past cues that would normally cause them to turn. We found that kinesin-5 is enriched in the transition (T-) zone of the growth cone and that kinesin-5 is preferentially phosphorylated on the side opposite the invasion of MTs. Moreover, when a growth cone encounters a turning cue, phospho-kinesin-5 polarizes even before the growth cone turns. Additional studies indicate that kinesin-5 works in part by antagonizing cytoplasmic dynein and that these motor-driven forces function together with the dynamic properties of the MTs to determine whether MTs can enter the P-domain. We propose that kinesin-5 permits MTs to selectively invade one side of the growth cone by opposing their entry into the other side.

Results and Discussion

Effects of Kinesin-5 Inhibition on Growth-Cone Turning

To test whether kinesin-5 (also called Eg5) is involved in growth-cone turning, we first used the "border assay," which utilizes a glass coverslip with a sharp border between a laminin-coated side and a polylysine-coated side. When explants of rat sympathetic ganglia are plated on the laminin side, growth cones normally turn when they reach the border. Indeed, almost all axons turned at the border in the case of growth cones of rat sympathetic ganglia are plated on the laminin side, growth cones normally turn when they reach the border. However, when monastrol was added to the culture medium, almost all axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5] (Figures 1 A–1C). Instead, the very few axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5] (Figures 1 A–1C). Instead, the very few axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5] (Figures 1 A–1C). Instead, the very few axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5] (Figures 1 A–1C). Instead, the very few axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5] (Figures 1 A–1C). Instead, the very few axons turned in the presence of monastrol, an allosteric inhibitor of kinesin-5 [5].

Next, we studied MT distribution in the bead assay. Growth cones of control neurons displayed a polarized MT array, with abundant MTs invading the P-domain on the side of the growth cone in the direction of the bead. There were fewer MTs invading the other side of the growth cone, away from the bead (Figures 1H and 1H). By contrast, the monastrol-treated neurons displayed no such bias on one side of the growth cone or the other with respect to MT distribution (Figures 11 and 11). The MT Distribution Ratio (MDR; see Supplemental Experimental Procedures, available online) was 2.43 in control growth cones (n = 13) and 1.10 in monastrol-treated growth cones (n = 14) (p < 0.009) (Figure 1 J). Analysis of the percentage of NGF-coated beads contacting axons that elicited filopodial sprouting [6] and the number of filopodia associated with each NGF-coated bead failed to reveal a difference, suggesting that monastrol did not alter the response of the filamentous actin to the NGF-coated beads (data not shown). These results suggest that kinesin-5 is crucial for the ability of growth cones to alter their MT distribution in response to a turning cue.

Microtubule Behaviors in Growth Cones after Experimental Manipulations of Kinesin-5 and Cytoplasmic Dynein

To visualize and quantify MT behaviors in growth cones, we quantified the number and velocity of the EGFP-EB3 "comets" within filopodia of EGFP-EB3-transfected neurons, as described previously [7–9]. Quantification over the course of 3 min showed significantly more comets (with a higher velocity) in filopodia of kinesin-5-depleted growth cones as...
Figure 1. Inhibition of Kinesin-5 Inhibits Growth-Cone Turning at a Laminin-Polylysine Border, Inhibits Turning toward NGF-Coated Beads, and Prevents Reorganization of MTs in Response to NGF-Coated Beads

(A and B) Axons (green, tubulin) grow on laminin (red), and their growth cones reach the border. (A) Growth cones of control explants treated with 0.1% DMSO turn at the border. (B) In the presence of 100 μM monastrol, most growth cones cross the border. Scale bar represents 15 μm.

(C) Quantification of the ratio of MT fluorescence on polylysine (axons that crossed the border) and laminin (axons that turned at the border) shows a significant increase in cultures treated with monastrol (red bar) compared to DMSO (blue bar). Asterisk denotes p ≤ 0.0001. Data represented as mean ± SEM.

(D) Quantification of the ratio of MT fluorescence, as above, shows that when kinesin-5 was depleted with the use of siRNA, a result similar to that obtained with monastrol treatment was observed. Asterisk denotes p ≤ 0.001. Data represented as mean ± SEM.

(E and F) Time-lapse phase-contrast images of growth cones of chicken DRG explants treated with DMSO (control) or monastrol for inhibition of kinesin-5. (E) Example of a growth cone, treated with 0.1% DMSO, turning toward the NGF-coated bead. (F) Example of a growth cone, treated with 100 μM monastrol, that had contacted the NGF-coated bead but did not turn toward the bead. Scale bar represents 10 μm.

(G) Percentage of growth cones that turned toward the NGF-coated bead. Asterisk denotes p < 0.001.

(H and I) Examples of MT (green) and actin (red) organization in a DMSO-treated growth cone (H and H') and a monastrol-treated growth cone (I and I') contacting an NGF-coated bead. The axonal axis (see Supplemental Experimental Procedures) is depicted as a blue line. Bead locations are shown as dotted circles denoting the circumference of the beads. The control growth cone shows an increased MT distribution toward the contact point with the bead, but the monastrol-treated growth cone does not show any marked asymmetry in MT distribution.

(J) Quantification of the MDR (as defined in Supplemental Experimental Procedures) in control and monastrol-treated growth cones. The blue bar indicates the MDR in control growth cones that contact the bead. The red bar indicates the monastrol-treated growth cones that contact the bead. The yellow and green bars indicate growth cones that do not contact beads in the cases of control and monastrol-treated growth cones, respectively. Asterisk denotes p < 0.009. Scale bar represents 10 μm. Data represented as mean ± SEM.
compared to control growth cones. These results, shown in Figure S1, suggest that kinesin-5 normally opposes the entry of MTs into filopodia.

Recent studies have shown that forces generated by cytoplasmic dynein enable MTs to overcome myosin-II-based retrograde flow and enter the P-domain, which includes the filopodia [7–9]. Forces generated by kinesin-5 have been shown to oppose forces generated by cytoplasmic dynein during mitosis [10] and in MT asters [11]. To investigate whether the same is true in growth cones, we treated rat sympathetic neurons with control siRNA or dynein heavy chain (DHC) siRNA for 2 days as described previously [12], then replated the neurons and allowed them to grow axons anew for 7 hr in either DMSO or monastrol. Under these conditions, DHC levels were reduced by roughly 90%, as shown by western blot [12]. After replating, the cultures were exposed to either monastrol or DMSO.

In results similar to those obtained in the siRNA-based depletion studies, quantification of the number of comets that invaded per filopodium (Figure 2E) during the course of 3 min showed a significant increase in of monastrol-treated growth cones (22.78 ± 2.72, n = 9) as compared to DMSO-treated growth cones (6.4 ± 0.86, n = 15) (p ≤ 0.001, two-tailed t test) (Figures 2A and 2C). In the case of DHC-depleted neurons, the number of comets was 1.3 ± 0.51 (n = 12) in the presence of the DMSO and increased nearly 13-fold, to 16.55 ± 1.01 (n = 11), in the presence of monastrol (p ≤ 0.001, two-tailed t test; Figures 2B, 2D, and 2E). Also, the velocity of EB3 comets in the filopodia (Figure 2F) was increased in monastrol-treated growth cones (0.0357 ± 0.005 μm/sec) compared to control growth cones (0.014 ± 0.003 μm/sec) (p ≤ 0.005, two-tailed t test). When dynein-depleted neurons were treated with monastrol, EB3 comet velocity was higher (0.023 ± 0.005 μm/sec) than comet velocities measured in growth cones of neurons treated with either control or DHC siRNA alone (0.0065 ± 0.002 μm/sec) (p ≤ 0.01, two-tailed t test). These results are consistent with a mechanism wherein kinesin-5 and cytoplasmic dynein work in an antagonistic relationship to regulate MT entry into filopodia. A similar antagonistic relationship is observed in measurements of axonal length (see Figure S2).

Several earlier studies demonstrated that the dynamic properties of MTs are important for determining whether they can enter filopodia during growth-cone turning [13–16]. We analyzed the relative roles of motor-driven forces and MT dynamics in determining MT distribution in growth cones by determining the MT levels in the P-domain of growth cones that had been treated with 5 nM vinblastine after depletion of dynein by siRNA or inhibition of kinesin-5 with monastrol. At this low concentration, vinblastine dampens MT dynamics without causing detectable MT loss [7]. The results show that no matter the experimental manipulation of DHC or kinesin-5, treatment with vinblastine results in a statistically indistinguishable low level of MTs in the P-domain (see Figure S3). These results suggest that forces generated by molecular motors make up one element in a multitiered mechanism that also includes the dynamic properties of the MTs.

The fact that MTs are able to overcome the retrograde flow of actin when both cytoplasmic dynein and kinesin-5 are suppressed may indicate that the assembly properties of the MTs...
are sufficient for overcoming the retrograde flow as long as the forces generated by the two opposing motors are eliminated. Alternatively, there may be other motors that contribute to the capacity of the MTs to overcome the retrograde flow. During mitosis, MTs are regulated by a variety of motor-driven forces that act together with the dynamic properties of the MTs [17], and the same might be true of growth cones.

Distribution and Phosphorylation of Kinesin-5 in the Growth Cone
Kinesin-5 is a homotetrameric motor protein that functions by generating forces between neighboring MTs [18]. In mitosis, the interaction of the motor protein with MTs is promoted by phosphorylation at amino acid 927 [19, 20]. To investigate whether phosphorylation at this site regulates kinesin-5 in growth cones, we conducted studies with an antibody that recognizes total kinesin-5 and an antibody that recognizes only phospho-kinesin-5. Virtually all growth cones showed prominent staining for total kinesin-5, whereas the intensity of staining for phospho-kinesin-5 varied. Our general sense was that axons that had been growing straight displayed the least phospho-kinesin-5 in their growth cones, whereas axons that were in the process of turning showed the most phospho-kinesin-5. Figures 3A and 3B show micrographs of growth cones double-labeled for MTs with each of the two kinesin-5 antibodies. Immunostaining with the total kinesin-5 antibody shows that kinesin-5 is present in the axonal shaft and diffusely throughout the growth cone but is strongly enriched in the T-zone (Figure 3A). Staining with the total kinesin-5 antibody shows a significantly higher ratio of kinesin-5 to MTs in the T-zone (0.744 ± 0.11) as compared to the C-domain (0.145 ± 0.03) (p ≤ 0.005, two-tailed t test).

Figure 3. Distribution and Phosphorylation of Kinesin-5 in Growth Cones
(A and B) Fluorescence micrographs of growth cones stained with total kinesin-5 antibody (A) and phospho-kinesin-5 antibody (B). (A) Growth cone stained for total kinesin-5; (A') the same growth cone, stained for MTs; and (A')' the overlay of A and A'. (B) Growth cone stained for phospho-kinesin-5; (B') the same growth cone, stained for MTs; and (B'') the overlay of kinesin-5 and MT. The box in (B) is shown in higher magnification in "P." Green arrowheads in "P" show the MTs in the P-domain, and the red region shows phospho-kinesin-5. The blue squiggly line indicates the perimeter of the P-domain. Scale bar represents 5 μm.

(C) Quantification of the polarized distribution of kinesin-5 in growth cones. The ratio of one side of kinesin-5 levels to the other is shown for growth cones immunostained with antibodies for total kinesin-5 (brown bar) and phospho-kinesin-5 (orange bar). Asterisk denotes p ≤ 0.01. Data represented as mean ± SEM.

(D) Quantification of the levels of kinesin-5 in the T-zone, C-domain, and axonal shaft. The values are ratioed against the levels of MTs in the same region.

(E) Quantification of the levels of phospho-kinesin-5 and MTs on each half of the growth cones. Red bars indicate the levels of phospho-kinesin-5, and green bars indicate the levels of MTs on that side of the growth cone in the P-domain. Single asterisk denotes p ≤ 0.05; double asterisk denotes p ≤ 0.005. Data represented as mean ± SEM.
and the axonal shaft (0.222 ± 0.03, n = 5) (p ≤ 0.05, two-tailed t test). The ratio of phospho-kinesin-5 to MTs was also higher in the T-zone (1.48 ± 0.37) as compared to the C-domain (0.138 ± 0.012) (p < 0.05, two-tailed t test) and the axonal shaft (0.099 ± 0.02, n = 6) (p ≤ 0.05, two-tailed t test). Also, in the T-zone, the ratio of the phospho-kinesin-5 to MTs is much higher than the ratio of total kinesin-5 to MTs. This suggests that a far greater proportion of the kinesin-5 in the T-zone is phosphorylated, as compared to the kinesin-5 in the axonal shaft and the C-domain (Figure 3D).

Whereas the total kinesin-5 antibody stained throughout the T-zone, the phospho-kinesin-5 antibody tended to stain more strongly in the T-zone on one of the growth-cone sides (Figure 3B). To quantify this, we divided the growth cone into equal halves and calculated the ratio of fluorescence intensity between the two halves. The ratio for total kinesin-5 is 1.15 (n = 10), showing a slight tendency to polarize, whereas the ratio for phospho-kinesin-5 is 1.74 (n = 9), showing a stronger tendency to polarize (Figure 3C) (p ≤ 0.01, two-tailed t test). Of particular interest, when we quantified MT levels on each half of the growth cones, we found that the half that showed an enrichment of phospho-kinesin-5 (832.49 ± 110.61) showed lower levels of MTs entering the adjacent P-domain (249.04 ± 48.86) as compared to MT levels in the P-domain on the other half of the growth cone (485.11 ± 80.44, n = 9) (p < 0.05, two-tailed t test) that showed lower levels of phospho-kinesin-5 (259.55 ± 52.66) (p ≤ 0.005, two-tailed t test) (Figure 3E, see also “P” in Figure 3B).

On the basis of these observations, we hypothesize that kinesin-5 is mainly nonphosphorylated before the growth cone is challenged to turn and after a turn has been completed but becomes phosphorylated when the growth cone is presented with a turning cue. If this is correct, polarization of phospho-kinesin-5 should occur even before the MT array becomes polarized and before the growth cone displays morphological indications of a turning response. To test these predictions, we analyzed the localization of phospho-kinesin-5 in a modified border assay (see Supplemental Experimental Procedures). We chose axons that had been growing straight toward the border but (1) had not yet reached it, (2) had just reached the border but showed no indication of turning yet, or (3) had completed turning in response to the border. As predicted, the first (n = 13) and third (n = 19) categories displayed very little phospho-kinesin-5 in their growth cones (Figures 4A and 4C). In sharp contrast, and also as predicted, prominent and highly polarized staining for phospho-kinesin-5 was detected in the second category of growth cones (n = 16, p ≤ 0.001, chi-square test) (Figure 4B; see Figure 4D also). These observations are consistent with a mechanism by which kinesin-5 is phosphorylated in a polarized fashion when the growth cone is challenged to turn, a critical event for the subsequent polarization of the MT array underlying growth-cone turning (Figure 4E).

In conclusion, the studies reported here demonstrate that kinesin-5 plays an essential role in polarizing the MT array in response to cues that cause the growth cone to turn.
Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, and six movies and can be found with this paper online at http://www.current-biology.com/supplemental/S0960-9822(08)01501-7.

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References

Supplemental Data

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Vidya C. Nadar, Andrea Ketschek, Kenneth A. Myers, Gianluca Gallo, and Peter W. Baas

Supplemental Experimental Procedures

Border Assay. Glass Coverslips (Bellco 15 X 15) were prepared for the border assay using modifications of the methods described previously [1]. For this, a CoverWell perfusion chamber gasket (Invitrogen) was used to create a sharp border between a region of polylysine and a region of laminin. The coverslips were flamed and the chamber gasket was pressed on one half of the coverslip. The open side was coated with polylysine at 37°C for 24 hours. The polylysine-coated side was rinsed with ddH2O three times and the coverslip was allowed to dry. A new gasket was then aligned with the first gasket on the polylysine-coated side to make sure that there was no gap between the two gaskets. The first gasket was removed from the uncoated side of the coverslip, which was then coated with laminin for 24 hours overnight at 37°C. The next day, the laminin was removed; the coverslip was rinsed with ddH2O three times and then allowed to dry. 800 μl of the cell culture medium containing either DMSO or monastrol was added to the coverslip. The explants, obtained from rat superior cervical ganglia (P0) or chicken DRGs (E7), were placed on the laminin side of the coverslip, approximately two-explants distance away. In the case of the siRNA experiments, dissociated neurons were obtained and transfected with control siRNA or kinesin-5 siRNA. The cells were re-suspended in cell-culture medium and 25 μl of cell suspension was placed as small droplets on the laminin side of the coverslip. 775 μl of medium was added to the coverslip after one hour, gently so as to not
disturb the attached cells. The explants or dissociated neurons were allowed to grow axons until their growth cones reached the border and either crossed or turned, which typically took 24-48 hours, depending on the distance from the border. The cultures were then fixed and fluorescently stained to reveal MTs and laminin as described previously [2]. Fluorescence images were acquired using Axiovision 4.6 and used for quantification. Quantification was performed using Axiovison 4.6 software. An ‘aligned rectangle’ was drawn along the border and used to measure the mean fluorescence intensity of MTs on the laminin and polylysine sides. The same rectangle was aligned at the end of the image on the polylysine side to determine the background fluorescence and was subtracted from the fluorescence on the laminin and polylysine side to obtain the actual fluorescence values for MTs on the laminin and polylysine sides. The ratio of MT intensity per pixel$^2$ was obtained using these actual values. The quantification was performed after all discernible axons extending toward the border had either turned or crossed the border. A modified border assay as described previously [3] was used for immunostaining for phospho-kinesin-5 (in figure 4).

**Bead Assay.** Chicken DRGs were dissected from embryos as described previously [4]. The DRG explants were placed on laminin-coated glass coverslips and allowed to grow axons overnight in medium containing 0.05 ng NGF. For the monastrol experiment, 0.1% DMSO was added to the control cultures and monastrol was used at 100 μM. NGF-coated polystyrene beads were prepared as described previously [4]. NGF-coated beads were introduced into the culture after the axons were more than 100 μm long. The beads were allowed to settle for 15 minutes. Growth cones approaching beads were identified and their behavior was recorded in live-cell movies using phase-contrast optics as described previously [4]. Images were acquired every 10
seconds until the axon grew past the bead. In some experiments, cultures were fixed after 2 hours and prepared for fluorescence staining of MTs and actin as described previously [2].

**Analysis of MT Distribution in Growth Cones Contacting NGF-Coated Beads.** Control and monastrol-treated cultures exposed to NGF-coated beads were fixed and stained for MTs and filamentous actin. Sampling criteria were designed with the aim of analyzing growth cones that had contacted the bead (either filopodial or lamellipodial contact, as ascertained by phalloidin staining for actin) but not yet turned (did not exhibit obvious morphological asymmetry toward the bead were sampled). A line extending 10 μm connecting the apparent center of the growth cone neck to the center of the axonal MT array was drawn to determine the axonal axis. This line was then extended to divide the growth cone into two halves. A line perpendicular to the axonal axis was then drawn intersecting it at the center of the growth cone neck. The total MT intensity was then determined within each half of the growth cone. Similarly, the area of the two halves of the growth cone was also determined. The total MT intensity on the side of the growth cone contacting the bead was then expressed as a ratio to the measurement on the side of the growth cone not contacting the bead. We call this ratio the “MT Distribution Ratio” or MDR. Similar measurements were performed on the growth cones not touching the bead using the “left” side as the fictional bead-contact side (left side defined as the side of the growth cone that is to the left side of the axonal axis if the axonal axis is presented as perpendicular to the bottom of the image). The Welch t-test (one-tailed) was used to determine the significance. Beads along axons in the explant’s periphery were analyzed to determine sprouting of filopodia in response to beads as described previously [5].
Depletion of Kinesin-5 and Cytoplasmic Dynein by siRNA. We used the identical “smart pool” of siRNA for cytoplasmic dynein (Dharmacon) and the same kinesin-5 siRNA (Ambion) and (and the scrambled sequence as a control) as in our previous studies [2, 6]. As in these earlier studies, the siRNA was introduced using the Amaxa Nucleofector after dissociation of the ganglia. Neurons were plated on plastic dishes, and given 2-3 days for the protein to be depleted. At this point, the neurons were re-plated on glass coverslips as described before [2, 6]. Western blots confirmed that, as in the earlier studies, over 95% of the kinesin-5 and over 90% of the DHC were depleted using this procedure. Given that immunostains for these two motors produce distinctive patterns in growth cones, we visually confirmed the efficacy of the siRNA in the re-plated cultures using immunostains (DHC antibody, Santa Cruz Biotechnology and antibody for total kinesin-5, Abcam) (see supplemental figures 4 and 5).

Analysis of Axonal Length. Rat neurons were cultured and transfected with either control siRNA or siRNA for DHC. The transfected neurons were plated on polylysine-coated plastic dishes and re-plated after 48 hours onto polylysine and laminin-coated coverslips either in the presence of DMSO or monastrol. The axonal lengths were analyzed after 7 hours of re-plating as described previously [2].

EGFP-EB3 Imaging in Growth Cones. Rat neurons from the superior cervical ganglia were transfected with 15 μg of EGFP-EB3 to visualize the EB3 comets, as described previously [3]. The neurons were also transfected with either control siRNA or siRNA for DHC. The transfected neurons were plated on polylysine-coated plastic dishes. After 48 hours, the neurons were re-plated onto polylysine-coated coverslips. After 18-24 hours, laminin (25 μg/ml) was
added to the medium to promote the formation of robust growth cones. DMSO or monastrol was added to the medium 30 minutes prior to imaging. Images of the growth cones were obtained every second for five minutes at an exposure time ranging from 300 ms to 700 ms and 2 x 2 binning. The images were quantified as described previously for the number of comets that entered the filopodia and the velocity of the comets [3].

**Vinblastine Studies.** Neurons were transfected with control or DHC siRNA, cultured for 2 days, and re-plated onto polylysine-coated coverslips as described above. After 3 hours, the neurons were treated with 25 μg/ml laminin to initiate axonal growth. After 2 more hours, the neurons were treated either with 5 nM vinblastine or DMSO for 30 minutes. These neurons were then treated with either DMSO or 100 μM monastrol for 30 minutes and then fixed and prepared for fluorescence staining of MTs and actin. The MT levels in the P-domain were analyzed as described previously [3] except that we defined the P-domain simply as the actin-rich distal region of each growth cone.

**Quantification of the Images Immunostained for Kinesin-5.** Fluorescence staining for kinesin-5 and MTs was performed after fixing in cold methanol, as described previously [7]. Polyclonal antibodies for total and phospho-kinesin-5 (Abcam) were raised in rabbit using synthetic non-phosphopeptide and phosphopeptide around the phosphorylation site of threonine 927. In some cases, neurons were triple-labeled to include staining for actin using an anti-actin antibody, to ensure proper identification of the borders of the P-domain. Fluorescence images were acquired using Axiovision 4.6. The growth cones were divided into two halves as follows: axonal axis was drawn by drawing a line form the apparent center of the growth cone neck to the
center of the axonal MT array. This axis was then extended to divide the growth cone into two halves. Using the outline spline tool, the actin immunostained region was traced on each half of the growth cones to identify the distal region of the growth cone (including the T-zone and P-domain). In some cases, the distal region was divided into two by drawing a horizontal midline through the traced actin-rich region to divide it into the P-domain and the T-zone. Fluorescence levels were determined for kinesin-5 and MTs in each half of the distal region of the growth cone and subtracted from the background fluorescence values to obtain the actual fluorescence values. These values were normalized over the total area and then used to obtain the ratio of kinesin-5 on each side of the growth cones. Similarly, levels of kinesin-5 and MTs were determined in the C-domain (region of the growth cone that was not stained for actin) and axonal shaft. These quantifications were carried out for growth cones that were immunostained for total kinesin-5 and phospho-kinesin-5.

The cultures in the modified border assay (experiment shown in figure 4) were immunostained for phospho-kinesin-5, MTs and laminin. All of the fluorescence images for phospho-kinesin-5 were taken using the same exposure time of 300 ms. The growth cones were classified into three categories as follows: an apparent axonal axis was drawn by drawing a line that connects the apparent centre of the growth cone neck and the center of the axonal MT array. If the axonal axis was parallel to the laminin/polylysine border, the growth cones were classified as having turned. The growth cones whose axonal axis was not parallel to the laminin/polylysine border, were classified as growth cones that had reached the border or as growth cones that had not reached the border, by analyzing whether the leading edge of the growth cone had or had not contacted the border, respectively. The number of growth cones that showed localization of
phospho-kinesin-5 was quantified in each category. Growth cones were defined as showing phospho-kinesin-5 localization when the measured gray scale values of phospho-kinesin-5 immunofluorescence in the fixed growth cone samples were higher than the background immunofluorescence values. The percentage of growth cones that showed a localization of phospho-kinesin-5 in each group was plotted and the chi-square test was carried out to test significance.
Figure S1. Quantification of EGFP-EB3 Comets in Control and Kinesin-5-Depleted Growth Cones. (A) Quantification of the number of comets that enter filopodia. Quantification over the course of 3 minutes showed significantly more comets in filopodia of kinesin-5-depleted growth cones (39.33 ± 2.64, n=6) as compared to control growth cones (7.66 ± 1.05, n=8) (p ≤ 0.0001, two-tailed t-test) (5-fold increase). (B) Quantification of the velocity of the comets that enter filopodia. The velocity of EB3 comets was greater after kinesin-5 depletion (0.125 ± 0.002 μm/sec) as compared to control neurons (0.043 ± 0.003 μm/sec) (p ≤ 0.001, two-tailed t-test). Red bar indicates control siRNA and yellow bar indicates kinesin-5 siRNA transfected growth cones. **, p ≤ 0.001.
Figure S2. Quantification of Axonal Length. Axonal length of dynein-depleted neurons (n=20) (blue bar) decreased by 51% (from $282 \pm 34.8 \, \mu m$ to $143 \pm 17.1 \, \mu m$, $p \leq 0.05$, two tailed t-test) compared to control neurons (n=17) (red bar). Monastrol treatment (n=22) (yellow bar) increased axonal length by 213% (from $282 \pm 34.8 \, \mu m$ to $600 \pm 76.8 \, \mu m$, $p \leq 0.05$, two tailed t-test). Treatment of dynein-depleted neurons with monastrol (n=26) (green) increased their length by 224% compared to the dynein-depleted neurons treated with vehicle alone (from $143 \pm 17.1 \, \mu m$ to $321 \pm 63.5 \, \mu m$, $p \leq 0.05$, two tailed t-test). *, $p \leq 0.05$. 
Figure S3. Quantification of MT Distribution in the P-Domain of Growth Cones Treated with Vinblastine. The graph shows the distribution of MTs in the P-domain of growth cones that has been normalized over the growth cone area. The distribution of MTs in the P-domain of growth cones in control siRNA transfected neurons treated with vinblastine (18.52 ± 5.53, n=5, light red bar) is significantly less than the control siRNA transfected neurons treated with DMSO (36.87 ± 7.39, n=4, dark red bar) (p ≤ 0.01, two-tailed t-test). DHC siRNA transfected neurons treated with DMSO (20.64 ± 1.0, n=7, dark blue bar) and vinblastine (19.48 ± 2.24, n = 4, light blue bar) show decreased levels of MTs in the P-domain, similar to the control siRNA transfected neurons treated with vinblastine. Control siRNA transfected neurons exposed to monastrol and treated with vinblastine (20.55 ± 3.9, n=6, light yellow bar) also shows significantly decreased levels of MTs in the P-domain as compared to the control siRNA
transfected neurons exposed to monastrol and treated with DMSO (60.14 ± 2.6, n = 8, dark yellow bar) (p ≤ 0.01, two-tailed t-test) but similar to the other groups that are treated with vinblastine. DHC siRNA transfected neurons exposed to monastrol and treated with DMSO (49.1 ± 4.91, n = 5, dark green bar) shows significantly higher levels of MTs as compared to the DHC siRNA transfected neurons exposed to monastrol and treated with vinblastine (22.40 ± 3.71, n=7, light green bar) (p ≤ 0.01, two-tailed t-test). (*, p ≤ 0.01).
Figure S4. Depletion of Kinesin-5 Using siRNA in Cultured Neurons. siRNA treatment effectively depletes over 95% of the kinesin-5 protein from the neurons, as quantified by Western blots (not shown). Depletion of kinesin-5 from the growth cone is illustrated here by immunofluorescence. Fluorescence micrographs of growth cones of neurons transfected with control siRNA (A and A’) and kinesin-5 siRNA (B and B’). (A, B) Growth cones showing total kinesin-5 immunostaining. (A’, B’) Same growth cones stained to reveal the MTs. Control siRNA transfected growth cones show an enrichment of kinesin-5 in the T-zone (A) and kinesin-5 siRNA-transfected growth cone shows absence of kinesin-5 (B). Scale bar, 5 μm.
Figure S5. Depletion of DHC Using siRNA in Cultured Neurons. siRNA treatment effectively depletes over 90% of the DHC protein from the neurons, as quantified by Western blots (not shown). Depletion of DHC from the growth cone is illustrated here by immunofluorescence. Fluorescence micrographs of growth cones of neurons transfected with control (A and A’) and DHC siRNA (B and B’). (A, B) Growth cones immunostained with DHC antibody. (A’, B’) Same growth cones immunostained to reveal MTs. Scale bar, 5 μm.
Supplemental References


