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Localized Alteration of Microtubule Polymerization in Response to Guidance Cues

Terri-Ann N. Kelly^{1,2}, Yasuhiro Katagiri¹, Keri B. Vartanian¹, Pramukta Kumar², Inn-Inn Chen¹, William J. Rosoff², Jeffery S. Urbach², and Herbert M. Geller^{1,*}

¹Developmental Neurobiology Section, Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

²Department of Physics, Georgetown University, Washington, DC

Abstract

Inhibition of microtubule dynamic instability prevents growth cone turning in response to guidance cues, yet specific changes in microtubule polymerization as growth cones encounter boundaries have not been investigated. In this study, we examined the rate and direction of microtubule polymerization in response to soluble nerve growth factor (NGF) and immobilized chondroitin sulfate proteoglycans (CSPGs) by expressing enhanced GFPEB3 in rat pheochromocytoma (PC12) cells. GFP-EB3 comets were monitored in live cells using time-lapse epifluorescent microscopy. Using an automated tracking system, the rate of microtubule polymerization was calculated as the frame-to-frame displacement of EB3 comets. Our results demonstrate that the rate of microtubule polymerization is increased following NGF treatment, while contact with CSPGs decreases microtubule polymerization rates. This reduction in microtubule polymerization rates was specifically localized to neurites in direct contact with CSPGs, and not at non-contacting neurites. Additionally, we found an increase in the percentage of microtubules polymerizing in the retrograde direction in neurites at CSPG boundaries with a concomitant decrease in the rate of retrograde microtubule polymerization. These results implicate localized changes in microtubule dynamics as an important component of the growth cone response to guidance cues.

Keywords

axonal guidance; CSPG; EB3; particle tracking; PC12 cells; NGF

Growing axons respond to positive and negative guidance cues, both soluble and substrate-bound, as they navigate to their targets (Tessier-Lavigne and Goodman, 1996). This response is coordinated by the growth cone, a specialized region at the tip of the growing axon. Yet the mechanisms by which growth cones and axons steer in response to guidance cues is not clear. Many studies have shown that the response to guidance cues requires a dynamic cytoskeleton (Buck and Zheng, 2002; Challacombe et al., 1996; Challacombe et al., 1997; Suter and Forscher, 1998). Early evidence suggested that the actin cytoskeleton controlled growth cone motility while the microtubules controlled axonal extension (Letourneau and Ressler, 1984; Marsh and Letourneau, 1984; Yamada et al., 1970), but it is now believed that actin-microtubule interactions are important as well (Challacombe et al., 1997; Lee and Suter, 2008; Buck and Zheng, 2002; Suter et al., 2004). For example, disruption of microtubule

*Corresponding Author: Herbert M. Geller, Ph.D. Developmental Neurobiology Section Cell Biology and Physiology Center National Heart, Lung, and Blood Institute National Institutes of Health 9000 Rockville Pike Bethesda, MD 20892 Phone: 301-451-9440 FAX: 301-594-8133 geller@helix.nih.gov.

dynamic instability alters both the direction of neurite growth (Bamburg et al., 1986; Tanaka et al., 1995) and the response to boundaries (Challacombe et al., 1997), and excessive microtubule stability causes pathfinding defects (Hendricks and Jesuthasan, 2009). These data indicate microtubules must play an important role in regulating the response to guidance cues.

A recent paper has demonstrated that exposure to Wnts changes microtubule polarization in growth cones (Purro et al., 2008), suggesting that guidance cues may produce localized changes in microtubule dynamics. In fact, all the proteins necessary for control of microtubule dynamic instability, including those involved in promoting polymerization, stabilization, destabilization and microtubule severing (Poulain and Sobel, 2009) are present in neurites and growth cones. Other studies have directly demonstrated microtubule dynamic instability in growth cones (Tanaka and Kirschner, 1995).

In this study, we evaluated the effects of positive and negative guidance cues on localized microtubule polymerization in PC12 cells, a valuable model of neurite outgrowth and neuronal differentiation, through the use of the EB3 surrogate marker system (Stepanova et al., 2003). NGF, in addition to its roles in survival and differentiation, serves as a strong positive guidance cue for many neuronal phenotypes (Rosoff et al., 2004; Cao and Shoichet, 2001). CSPGs provide repulsive guidance cues to developing and regenerating axons (Katoh-Semba et al., 1995; Laabs et al., 2007). We now provide evidence that NGF and CSPGs have opposite effects on the rate of microtubule polymerization: causing an increase or decrease, respectively. Significantly, the effect of CSPGs on microtubule polymerization rate was localized to the neurites in direct contact with CSPGs; other neurites of the same cell were unaffected. These data suggest that local signaling and alterations in microtubule dynamic instability are important components of the response of neurons to guidance cues.

MATERIALS AND METHODS

DNA Construct

RT-PCR was performed to obtain human EB3 cDNA with the following primers (5'-AAGCTAGCCG GAGCCGCCTC GGC -3' and 5'-TTAAGCTTGT ACTCGTCCTG GTCTTCTTGT TGATGC-3'). The PCR product was subcloned into NheI/HindIII sites of pEGFP-N1 (Clontech, Mountain View, CA), generating pEGFP-EB3 plasmid.

Cell Culture and Transfection

PC12 cells were maintained as described previously (Katagiri et al. 2000). PC12 cells were transfected using Nucleofector V solution (Lonza, Gaithersburg, MD) according to the manufacturer's protocols. After transfection, the cells were cultured for 2–3 days on poly-L-lysine (PLL)-coated glass-bottomed dishes (Mat-Tek, Ashland, MA) in DMEM containing 7.5% fetal bovine serum, 7.5% horse serum, penicillin, streptomycin, and 100 ng/mL NGF. In some experiments, cells were maintained in NGF-free media.

CSPG boundaries were created as previously described (Wang et al., 2008; Laabs et al., 2007). A CSPG-PLL interface was created by placing a 5 μ L drop of 5 μ g/ml chicken CSPGs (Millipore, Billerica, MA) and Texas Red (Invitrogen, Carlsbad, CA) in the center of a PLL-coated coverslip. Texas Red was used to visualize the CSPG-rich areas. As a negative control, Texas Red and Hanks' Balanced Salt Solution (HBSS) were used to create a similar region. Ten to twenty thousand PC12 cells were plated into each 35 mm dish and maintained for 2–3 days, as indicated above.

Live Cell Imaging

Two to three days after GFP-EB3 transfection and NGF-treatment, PC12 cultures were transferred to a heated stage on a Nikon Eclipse TE2000 microscope and maintained at 37°C in 5% CO₂ in air. Cells selected for observation were chosen based on the obvious presence of a well-defined growth cone with active filopodia. Time-lapse images were acquired at 0.8 sec intervals, using a 100× NA 1.4 oil objective with a Hamamatsu Orca ER camera (0.066 μm/pixel). All devices were controlled by MetaMorph imaging software (Molecular Devices, Sunnyvale, CA).

Particle Tracking and Image Analysis

A flow chart of the overall image processing methodology is presented in Figure 1A. As outlined, time-lapse image stacks were preprocessed prior to analysis to allow for successful particle tracking. First, each frame was normalized by the total intensity (sum of all pixel values) to correct for photobleaching. A drift correction algorithm was then used to compensate for the motion of the cell. Next, a constant background was subtracted off of each frame, resulting in a dark image with bright EB3 comets. Finally, a real-space bandpass filter which suppresses pixel noise and large-scale image variations while retaining information on length scales was applied to enhance the comets and suppress background noise. Each of these routines are included in a modified version of Crocker and Weeks' particle tracking software (Figure 1B; <http://www.physics.emory.edu/faculty/weeks/idl/>) that was used to generate a spreadsheet of comet trajectories. Only comets that could be tracked for 7 or more frames (5.6 sec or more) were included in the analysis. The data was then exported to ImageJ (available at <http://rsb.info.nih.gov/ij/>) for further analysis. The validity of the tracks was verified using a custom ImageJ plug-in, which creates a 2D projected image of the time-lapse sequence (Figure 1C; see also Supplementary Movie 1) onto which the tracked comets are overlaid (Figure 1D; see also Supplementary Movie 1). The MTrackJ plug-in (<http://www.imagescience.org/meijering/software/mtrackj/>) was also used to visualize the tracks. Trajectories where the ratio of the total distance traveled to the net displacement of the trajectory was <0.5, were considered most likely noise and not used in further calculations. In addition, trajectories identified as being localized to filopodia were discarded because of potential movement artifacts. The remaining trajectories were then classified as either retrograde or anterograde and according to their position within the cell. The frame-to-frame displacement of the EB3 comets was determined for each step in every microtubule trajectory, and the instantaneous microtubule polymerization rate was calculated by dividing this displacement by the time between frames. The average polymerization rate for each microtubule trajectory was calculated as the sum of the displacements divided by the duration of the trajectory.

To quantify the percentage of microtubules polymerizing in the anterograde and retrograde directions, the primary axis of fan-shaped growth cones was determined by visual inspection. Anterograde polymerizing microtubules were defined as microtubules in growth cones and neurites that were polymerizing towards the leading edge (away from the cell body) at an angle that was within $\pm 60^\circ$ of the primary axis, whereas retrograde polymerizing microtubules were moving away from the leading edge of the neurites at an angle that is within $\pm 60^\circ$ of the primary axis. The microtubule polymerization rates were calculated for each group of tracks, i.e., anterograde and retrograde, as indicated above. Additionally, the MultipleKymograph plug-in for ImageJ was used to generate kymographs of comet movements. Brightness, contrast and gamma were adjusted in Photoshop to improve clarity.

Statistical Analysis

Statistical analysis was done using the InStat software (GraphPad Software, La Jolla, CA), where $p < 0.05$ was considered significant. Parametric and non-parametric (Kruskal-Wallis

Test) ANOVA were used as appropriate. The values presented in the results section are mean $\pm$ SEM. SigmaPlot software (Systat Software, San Jose, CA) was used to generate cumulative frequency histograms and box and whisker plots of the microtubule polymerization rates. Because histograms demonstrated that rates were not normally distributed, the non-parametric Wilcoxon-Mann-Whitney test was used to determine significant differences in the histograms.

RESULTS

The level of GFP-EB3 expression varied between PC12 cells, with the highest expression resulting in the continuous decoration of microtubules. Therefore, only cells displaying levels of GFP-EB3 expression that produced clearly defined comets were selected for recording (Supplementary Movie 1). Time-lapse images were obtained at 0.8 sec intervals. This interval was selected because it enabled us to determine the displacements of the EB3 comets using an automated technique for particle tracking. At longer time intervals, the displacements of the comets were too large for the program to continually track the same comet with high fidelity. Data analysis was limited to 40 frames per sequence, which resulted in identification of anywhere between 20–170 trajectories per sequence; longer sequences led to failure of the program due to the large number of trajectories that had to be tracked. We validated the data obtained via the automated tracking program using a custom ImageJ plug-in, the MTrackJ plug-in, and by manually assessing each track individually to verify the consistency and repeatability of the results (data not shown). Moreover, our results are consistent with previous reports of EB comet analyses in neurons that yielded average microtubule polymerization rates of 10–26 $\mu\text{m}/\text{min}$ (Ma et al., 2004; Morrison et al., 2002; Stepanova et al., 2003).

Microtubule polymerization rates are increased by NGF-treatment

NGF promotes neurite extension in PC12 cells and primary neurons (Greene and Tischler, 1976; Levi-Montalcini and Cohen, 1956), and soluble NGF can serve as a guidance molecule for neurite growth (Rosoff et al., 2004). Because NGF-induced neurite extension in PC12 cells requires tubulin polymerization (Letourneau and Ressler, 1984), and increases the level of polymerized tubulin (Black et al., 1986), we first compared the rates of microtubule polymerization in the presence and absence of NGF. As expected, NGF promoted neurite formation in these cells (compare Figure 2A to Figure 2B; see Supplementary Movie 2 and 3, respectively for corresponding image sequences). Quantitative analyses of GFP-EB3 comets in 25 NGF-treated and 24 untreated PC12 cells indicate that NGF-treatment significantly increases microtubule polymerization rates (Figure 2C; $p < 0.001$). In untreated, NGF-naïve samples, the average microtubule polymerization rates were $13.4 \pm 0.1 \mu\text{m}/\text{min}$ versus $16.2 \pm 0.2 \mu\text{m}/\text{min}$ for cell bodies of NGF-treated cells. We next compared the rate of polymerization in the soma and the neurites of the cell. In NGF-treated PC12 cells, the microtubule polymerization rates were significantly slower in the neurites compared to the cell bodies ($16.2 \pm 0.2 \mu\text{m}/\text{min}$ in the cell bodies versus $14.9 \pm 0.2 \mu\text{m}/\text{min}$ in the neurites; $p < 0.001$).

In axons, about 90% of microtubule polymerization is in the anterograde (away from the cell body) direction, while up to 35% of microtubules in dendrites polymerize in the retrograde (towards the cell body) direction (Ma et al., 2004; Stepanova et al., 2003). We therefore examined the direction of comet trajectories in the cell bodies and the neurites, as outlined in Figure 2B. In cell bodies, the direction of microtubule polymerization is random (insets in Figure A, B). This randomness was confirmed using kymograph analysis of these cells (Figure 2D, E), while the microtubules in the neurites of NGF-treated cells are primarily polymerizing toward the leading edge of the neurites (Figure 2F), consistent with an axonal phenotype (Black et al., 1986).

Localized decrease in microtubule polymerization rates at CSPG boundaries

In order to determine the effects of immobilized CSPGs on microtubule polymerization, live cell imaging was conducted on GFP-EB3 transfected PC12 cells whose neurites encountered CSPG- and HBSS-coated regions (Figure 3). As we have previously shown in neurons (Laabs et al., 2007), the neurites crossed into the HBSS-coated regions (Figure 3A), but did not cross the interface with CSPGs (Figure 3B). The rates of microtubule polymerization were analyzed in three regions of the PC12 cells: cell bodies, neurites away from the interface, and neurites contacting the interface (Figure 3). We analyzed tracks from 18 NGF-treated cells at CSPG spots, and 24 cells at HBSS spots. As observed in NGF-treated cells in Figure 2, the microtubule polymerization rates were significantly slower in the neurites compared to cell bodies ($p < 0.05$).

We compared the polymerization rates in neurites that were in direct contact with spots with rates in neurites that did not. We observed that microtubule polymerization rate was significantly reduced only in neurites that were in direct contact with the CSPGs (Figure 3C): the median rate of microtubule polymerization was 12.4 $\mu\text{m}/\text{min}$ in the neurites at the spot, 15.0 $\mu\text{m}/\text{min}$ away from the spot, and 16.0 $\mu\text{m}/\text{min}$ in the cell body ($p < 0.001$). On the other hand, there were no differences in microtubule polymerization rates of neurites that were in direct contact with HBSS-coated regions compared to those away from the HBSS. Thus, contact with CSPGs has a highly localized action on microtubule polymerization.

We created cumulative frequency histograms of the microtubule polymerization rates in these neurites (Figure 3D, E). The distributions overlap in the regions where cells are not in contact with CSPGs. At CSPG interfaces, however, there is a significant increase in the percentage of slow moving comets with a corresponding decrease in the percentage of fast moving comets ($p < 0.01$). In PC12 cells that contact CSPG spots, the percentage of comets that move at less than 10 $\mu\text{m}/\text{min}$ is 11% in the cell bodies, 13% in the neurites that are not in contact with CSPGs, and 30% in the neurites that directly contact CSPGs. The corresponding percentages are 6, 7 and 8 for the same regions of cells contacting HBSS. Taken together, these results strengthen our conclusion that CSPG boundaries alter microtubule dynamics via localized decreases in microtubule polymerization rates.

Increase in retrograde polymerization at CSPG boundaries

Most microtubules in neurons are polarized with their +ends distally. The fraction of microtubules with reverse polarity was originally reported as <10% in axons (Burton and Paige, 1981) to nearly 50% in dendrites (Burton, 1988). Similarly, the vast majority of EB3 comet trajectories in primary neurons (80–90%) moved in the anterograde direction (towards the distal end of the neurite) in axons (Ma et al., 2004; Stepanova et al., 2003). We therefore examined the direction of microtubule polymerization in a subset of neurites at or away from CSPG interfaces. For each neurite, all the trajectories were classified manually as either anterograde (away from the cell soma), retrograde (towards the cell soma), or neither. An example of a typical growth cone with the direction of trajectory polymerization is presented in Fig. 4A. Fig. 4B presents an example of a control neurite, in which microtubules polymerize overwhelmingly in the anterograde direction (see also Supplementary Movie 4). We demonstrate this directionality with the use of kymograph analysis in Fig. 4D. When the percentage of microtubules polymerizing in the anterograde and retrograde direction were quantified (Figure 4F), we observed that in control neurites, $14 \pm 2\%$ of microtubules polymerized in the retrograde direction versus $81 \pm 3\%$ in the anterograde direction. In contrast, neurites that contacted CSPGs (Figure 4C; see also Supplementary Movie 5) displayed a significantly higher percentage of microtubules that polymerized in the retrograde direction (Figure 4E). At these boundaries, $35 \pm 3\%$ of the microtubules were polymerizing in the

retrograde compared to $64 \pm 3\%$ polymerizing in the anterograde direction ($p < 0.001$ as compared to control).

We also examined the rate of polymerization in neurites in the anterograde and retrograde directions (Figure 4G). We found that contact with the CSPGs resulted in a slowing of polymerization: while the rates of polymerization in neurites at control boundaries were almost equal, there was a significant decrease at CSPG boundaries. At HBSS spots, the median polymerization rates were $14.9 \mu\text{m}/\text{min}$ in the anterograde direction and 14.4 in the retrograde direction, while at CSPG boundaries the respective rates were 14.0 and 13.0 ($p < 0.05$). Thus, contact with CSPGs locally alters both the direction and rate of microtubule polymerization.

DISCUSSION

During neural development and after injuries to the nervous system, dynamic interactions with positive and negative guidance cues direct growing axons; however, the effect of these guidance molecules on microtubule polymerization is not well understood. In this study, we demonstrated that a positive growth factor (NGF) and a repulsive guidance cue (CSPGs) differentially alter the rate and direction of microtubule polymerization in neurites. NGF, a growth factor that induces directed neurite outgrowth (Rosoff et al., 2004), significantly increased the rate of microtubule polymerization. On the other hand, when neurites encountered immobilized CSPGs, the polymerization rate decreased, while neurites of the same cell that did not touch CSPGs were unaffected. We also showed that contact with CSPGs significantly increased the percentage of microtubules that polymerize in the retrograde direction and these microtubules polymerize more slowly than those in the anterograde direction. Together, these data support the existence of a local signaling mechanism by which guidance cues alter microtubule dynamics.

We found that the rate at which microtubules polymerize was significantly slower in the neurites than in the cell bodies, and was further reduced in neurites in contact with CSPGs. A similar difference between soma and neurites was observed in differentiated Neuro2A cells (Morrison et al., 2002), but an opposite result was obtained in primary *Xenopus* spinal neurons (Kim and Chang, 2006). The slower polymerization rate in neurites may simply reflect the stoichiometry of free tubulin, as the concentration of tubulin monomers is greatest in the soma, and decreases in the neurites (Miller and Joshi, 1996). However, the further reduction in polymerization rate when neurites contact CSPG boundaries implies the existence of localized signal transduction mechanisms that may influence neurite growth and guidance.

The relationship between microtubule polymerization and neurite outgrowth and axonal guidance is not strict. NGF-treated PC12 cells grow at a rate of $30\text{--}50 \mu\text{m}/\text{day}$ (Black and Greene, 1982), which is three orders of magnitude slower than the rate of microtubule polymerization. Thus, the rates of both polymerization and catastrophe must influence neurite growth. While we were unable to measure catastrophe, contact with a $5 \mu\text{g}/\text{ml}$ CSPG boundary reduced the rate of microtubule polymerization by 17%, from $15 \mu\text{m}/\text{min}$ to $12.4 \mu\text{m}/\text{min}$. Interestingly, this change was all along the neurite, suggesting that the inhibitory signal is propagated towards the cell body. When PC12 cells were grown on a uniform substrate of the same concentration of CSPGs, neurite outgrowth was inhibited 60% (Gopalakrishnan et al., 2008). This implies that neurons integrate signals from different processes to control overall neurite outgrowth. Moreover, inhibition of microtubule polymerization is likely not the only target of CSPGs in regulation of growth and guidance.

While no one membrane receptor for CSPGs has been identified, disruption of several different signal transduction pathways that affect microtubule dynamics abrogates their inhibitory actions (Dill et al., 2008; Gopalakrishnan et al., 2008). Most prominently, inhibition of Rho

GTPase or Rho kinase promotes axonal growth into inhibitory environments, *in vivo* as well as *in vitro* (Dergham et al., 2002; Dubreuil et al., 2003; Hirose et al., 1998; Ito et al., 2007; Lord-Fontaine et al., 2008; Gopalakrishnan et al., 2008), and increases PC12 neurite outgrowth on CSPGs (Gopalakrishnan et al., 2008). Growth cone turning due to the localized stabilization and disruption of microtubules can also be prevented by inhibition of Rho GTPases (Buck and Zheng, 2002). Blockade of protein kinase C (PKC) reduces the inhibitory activity of CSPGs (Powell et al., 2001; Sivasankaran et al., 2004), and modulation of PKC activity with phorbol esters increases microtubule polymerization in growth cones (Kabir et al., 2001). Additionally, CSPGs and myelin transactivate the EGF receptor in a calcium-dependent manner (Koprivica et al., 2005); EGF activation then increases Erk activity, which can alter microtubule stability (Harrison and Turley, 2001). It has recently been demonstrated that the response to CSPGs is diminished in neurons with a genetic deletion of the receptor tyrosine phosphatase σ (Shen et al., 2009). Whether these pathways are involved in the observed changes in microtubule polymerization rates is a question for further investigations.

In differentiated neurons, the polarity of microtubules is primarily anterograde in axons, but a significant fraction also have the opposite polarity in dendrites (Burton and Paige, 1981; Burton, 1988). More recent studies that track microtubule polymerization in real time demonstrate a similar preponderance of anterograde microtubule polymerization in axons, with up to 18% in the retrograde direction (Ma et al., 2004; Stepanova et al., 2003; Stone et al., 2008; Kim and Chang, 2006; Shemesh and Spira, 2010). We found that microtubules in processes of NGF-treated PC12 cells polymerized primarily in the anterograde (+end towards the growth cone) direction, with about 15% in the retrograde direction, consistent with these previous observations in axons. Interestingly, the neurites that touched CSPG spots had a higher percentage of retrograde polymerizing microtubules. There are several potential explanations. Microtubule nucleation has been traditionally thought to occur at the centrosome, with the +end towards the axon; however, there is increasing evidence for non-centrosomal nucleation in several different cell types, including neurons (Shemesh and Spira, 2010; Efimov et al., 2007; Keating and Borisy, 1999). Thus, nucleation could occur at the stalled neurites, causing retrograde polymerization. Alternatively, microtubules in paused growth cones form looped arrays (Schaefer et al., 2002; Dent et al., 1999), and it may be that the retrogradely polymerizing microtubules are extensions of these looped microtubules. Another possibility is that as the neurites sense the CSPG spot, microtubules are severed (Schaefer et al., 2002) and then continue to polymerize in a retrograde direction.

We developed and implemented automated GFP-EB3 comet tracking that utilized an 0.8 sec acquisition time. Our initial studies used 5 sec intervals, and manually tracked comets, but we found that the results were highly dependent on the observer, and relatively few tracks could be accurately analyzed. We also tried to acquire data using either TIRF or spinning-disk confocal techniques, but the trajectories were often untraceable due to movement in and out of the focal plane in the relatively thick PC12 growth cones. In order to accurately track comets and increase throughput, we adopted Crocker and Weeks' particle tracking program (<http://www.physics.emory.edu/faculty/weeks/idl/>) to automatically determine the change in position of the EB3 comets over time. Since this algorithm could not accurately track comets at 5 sec intervals, we progressively shortened the sampling interval and settled on the 0.8 sec interval as the longest interval that could be reliably tracked. This method is susceptible to noise; therefore, each trajectory was classified as to whether it fit the description of a polymerizing microtubule (relatively straight trajectory) (Stepanova et al., 2003). Future improvements in automated comet analysis will depend upon creating more reliable methods of identifying valid tracks. By incorporating these methodologies, we were able to more quickly and accurately determine the rates at which the microtubules polymerize, and increase the number of cells and microtubule tracks included in our analysis.

In summary, we demonstrate the rate of microtubule polymerization is increased by a positive growth factor (NGF) and decreased by a negative guidance cue (CSPGs), respectively. When neurites contact inhibitory CSPG spots, there is an increase in the percentage of microtubules polymerizing in the retrograde direction. These actions of CSPGs are restricted to neurites in direct contact with the spot, indicating that the signaling mechanisms controlling microtubule dynamics are localized to the growth cone. Identifying the receptors and the signal transduction pathways that control these processes is an important area of future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Automated image tracking determines microtubule polymerization trajectories in PC12 cells. A. Flow chart outlining the imaging processing methodology. Images were acquired at 800 ms intervals and stored. The images were then preprocessed to correct for photobleaching, drifting, and noise. A particle tracker program was used to identify GFP-EB3 comets in each frame and identify comet trajectories. The data was overlaid onto the image stacks to generate images of the trajectories of the comets. B. Particle identification in a frame of a sample growth cone. The coordinates of the centroid of the GFP-EB3 comets was identified in each frame based on size and intensity; these parameters were manually adjusted to maximize identification (circles). C. Z-projection of the EB3-GFP time-lapse stacks generates lines that represent

microtubule tracks. D. Identified trajectories are overlaid onto the z-projected image to visualize their locations and temporal sequence.

Figure 2. NGF increases microtubule polymerization rate in PC12 cells. A. Untreated PC12 cell. B. Differentiated PC12 cell showing partial cell body and neurites. (Scale bars = 10 μm). Insets illustrate tracks in the white boxed areas in cell bodies. C. Box and whisker plots of the microtubule polymerization rate in cell bodies of untreated PC12 cells and the cell bodies and neurites of NGF-treated PC12 cells (n = number of trajectories as indicated; ** $p < 0.001$). The rate of microtubule polymerization was significantly slower in the neurites of the NGF-treated PC12 cells compared to the cell bodies (* $p < 0.001$). Boxes are the upper and lower quartiles, whiskers are the maximum and minimum values and center line is the median value. D, E, F. Kymographs along lines shown in A and B confirm that microtubule polymerization direction is random in the cell bodies, while in the neurites the microtubules are primarily polymerizing in the anterograde direction, i.e., away from the cell body.

Figure 3.

Chondroitin sulfate proteoglycans (CSPGs) create a boundary that produced significant localized reductions in microtubule polymerization rate. A. Control (HBSS) region does not create a boundary, i.e. neurites are able to cross into the region. B. CSPG regions create a boundary that prevent neurite crossing. For analysis, comet trajectories are localized in three distinct regions, the cell bodies, neurites away from the spot (arrowheads), and neurites that are at or on spots (arrows). Scale bars are 10 μm . C. Box and whisker plots showing that microtubule polymerization rate is significantly reduced in neurites encountering CSPG spots (n = number of trajectories as indicated in figure, * $p < 0.05$ versus the cell bodies in HBSS encountering cells, ** $p < 0.001$ versus cell bodies and neurites away from the CSPG spots).

Boxes are the upper and lower quartiles, whiskers are the maximum and minimum values and center line is the median value. D, E. Cumulative frequency histograms of the rates of microtubule polymerization in the cell bodies and neurites away from and at HBSS spots (D) and cell bodies and neurites away from and at CSPG spots (E).

Figure 4.

Chondroitin sulfate proteoglycan (CSPG) spots significantly increase retrograde microtubule polymerization. A. Growth cone illustrating the classification of polymerization direction as either away from the cell body towards the the leading edge (anterograde) or towards the neurite shaft and cell body (retrograde). B. Neurite that is not at spot. C. Neurite at CSPG spot. Arrows in B and C point towards the cell bodies. The dashed white line in C shows the location of the CSPG spot. Scale bars are 10 μm. D – E. Kymographs of the regions centered on the yellow lines in B and C showing that, away from spots, the microtubules are polymerizing in the anterograde direction, and that at CSPG spots there is an increase in the percentage of microtubules polymerizing in the retrograde direction. In the right kymographs, green dotted lines overlay anterograde trajectories, while red lines overlay retrograde trajectories. (Duration = 32 sec. for each kymograph) F. Bars indicate the percentage of the microtubules polymerizing in either anterograde (green), retrograde (red) or neither (blue) direction. At CSPG boundaries, there is an increase in the percentage of microtubules polymerizing in the retrograde direction and a corresponding decrease in the percentage polymerizing in the anterograde direction (n = 12 – 15, ** $p < 0.001$). The data is presented as mean ± SEM. G. Box and whisker plots showing that the rate of microtubule polymerization was slower in the retrograde direction in neurites encountering CSPG-rich areas (n are the number of tracks as indicated in the figure, * $p < 0.05$, + $p < 0.01$). Boxes are the upper and lower quartiles, whiskers are the maximum and minimum values and center line is the median value.