Cytoskeletal Dynamics Underlying Collateral Membrane Protrusions Induced by Neurotrophins in Cultured *Xenopus* Embryonic Neurons

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ABSTRACT: The establishment and refinement of neuronal connections depend on dynamic modification of the morphology and physiology of developing axons in response to extrinsic factors. In embryonic cultures of *Xenopus* spinal neurons, acute application of brain-derived neurotrophic factor (BDNF) induced rapid collateral protrusion of filopodium-like microspikes and lamellipodia along the neurite processes, leading to a morphologic alternation of the neuron. Both types of membrane protrusions contained high concentrations of actin filaments and depended on the polymerization of the actin cytoskeleton. Immunofluorescent staining, however, revealed the presence of microtubules (MTs) in lamellipodia induced by BDNF. These MTs appeared to have arisen from debundling of MTs in the neurite shaft at the protrusion sites, splaying and extending in the rapidly protruding lamellipodia. Inhibition of microtubule polymerization by nocodazole largely abolished the formation of lamellipodia but not of microspikes. Taken together, our results suggest that collateral sprouting of microspikes and lamellipodia involve distinctly different cytoskeletal mechanisms. Although the actin cytoskeleton is solely responsible for microspike formation, cooperative efforts by microtubules and actin filaments are essential for lamellipodial protrusion in response to extrinsic factors. © 2002 Wiley Periodicals, Inc. J Neurobiol 54: 393–405, 2003

Keywords: **microtubule; actin; sprouting; lamellipodia; microspikes; membrane ruffling**

INTRODUCTION

Filopodia and lamellipodia are two motile structures at the nerve growth cone that are crucial to the growth and guidance of nerve fibers during development (Lockerbie, 1987; Bray and Hollenbeck, 1988; Smith, 1988; Goldberg and Burmeister, 1989). Filopodia and lamellipodia also play an important part in the structural plasticity of neuronal connections. The sprouting of new motile filopodia and lamellipodia from previously quiescent regions of a neuron is fundamental for the development of collateral branches (Bastmeyer et al., 1998) as well as for the dynamic morphologic changes involved in the refinement of neuronal connections (Robbins and Polak, 1988; Ziv and Smith, 1996; Fiala et al., 1998; Maletic-Savatic et al., 1999). There is ample evidence that the core structural component in motile filopodia and lamellipodia is the actin cytoskeleton, and its dynamic reorganization underlies the motile activity of filopodia and lamellipodia (Smith, 1988; Okabe and Hirokawa, 1991; Lin et al., 1994). Microtubules (MTs), on the other hand, are generally absent from these motile structures in non-neuronal (Heath and Holifield, 1991a,b) as well as neuronal (Smith, 1988) cells. It is believed that the actin-based motility in lamellipodia drives the forward movement of the cell or the growth cone fol-

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lowed by an "engorgement" process in which microtubules and other cellular components extend into the newly created cytosolic space to consolidate the new growth (Mitchison and Kirschner, 1988; Smith, 1988; Burmeister et al., 1991; Lin and Forscher, 1995). Recent studies, however, have indicated the existence of interactions between the actin and microtubule cytoskeletal systems and suggest that MTs may participate in many actin-based cellular events including motility (Goslin et al., 1989; Vega and Solomon, 1997; Waterman-Storer and Salmon, 1997). In nerve growth cones, individual MTs were observed to occasionally penetrate the actin-rich P-region to reach the leading edge of the lamellipodia, suggesting a potential role for MTs in growth cone navigation (Letourneau, 1983; Gordon-Weeks, 1991; Tanaka and Kirschner, 1991). Furthermore, MT-containing collateral spikes have also been observed previously (Goldberg and Burmeister, 1992; Gallo and Letourneau, 1998). Nevertheless, a causal relationship between microtubules and actin-based protrusive activities has not yet been established.

Neurotrophins are a family of neurotrophic factors with profound influences on the survival, differentiation, and neurite outgrowth of the nervous systems (Davies, 1994). Recent studies have demonstrated that neurotrophins also regulate axonal and dendritic sprouting and branching of developing and mature nervous systems (Diamond et al., 1992; Morfini et al., 1994; Cohen-Cory and Fraser, 1995; Patel and McNamara, 1995; McAllister et al., 1996; Gallo and Letourneau, 1998). In *Xenopus* cultures, neurotrophins exert a variety of acute and long-lasting effects on embryonic spinal neurons at various developmental stages, including the enhancement of survival and neurite outgrowth (Ming et al., 1997), potentiation of synaptic transmission (Lohof et al., 1993; Berninger and Poo, 1996), chemotropic and collapsing effects on growth cones (Ming et al., 1997; Wang and Zheng, 1998). Acute application of brain-derived neurotrophic factor (BDNF) to 1-day-old *Xenopus* neurons elicited extensive membrane protrusions at multiple locations along the quiescent neurite shaft as well as at the growth cone, leading to alternations of the cell morphology (Ming et al., 1997). In this study, we have further examined the membrane protrusions induced by acute application of BDNF. We have now characterized two primary types of membrane protrusions induced by exogenous BDNF: filopodium-like microspikes (hereafter referred to as *microspikes*) and lamellipodia. We then examined the cytoskeletal dynamics underlying these two types of membrane protrusions. We show that while the induction of both lamellipodia and microspikes by BDNF depended on the actin cytoskeleton, the formation of lamellipodia upon BDNF application also depended on MTs. These results demonstrate a differential involvement of the actin and microtubule cytoskeleton in microspikes and lamellipodia and may suggest a novel role for microtubules in actin-based lamellipodial activities elicited by extracellular factors.

MATERIALS AND METHODS

Cell Cultures

Embryonic *Xenopus* spinal neurons were prepared using the method reported previously (Spitzer and Lamborghini, 1976; Tabti and Poo, 1990). In brief, tissues of the neural tube were dissected from developing *Xenopus* embryos at stage 20–22 (Nieuwkoop and Faber, 1967), dissociated in a Ca^{2+}-Mg^{2+}-free Ringer's solution containing EDTA (115 m*M* NaCl, 2.5 m*M* KCl, 10 mM HEPES, and 0.5 m*M* EDTA, pH 7.6) for 20–25 min, plated on clean 40 × 22-mm No.1 glass coverslips (VWR Scientific, West Chester, PA) that were placed in Falcon 50 × 9-mm style Petri dishes (Becton Dickinson Co., Lincoln Park, NJ), and incubated at room temperature (20–22°C). The culture medium consisted of 50% (vol/vol) Leibovitz medium (Life Technologies, Gaithersburg, MD), 49% (v/v) Ringer's solution (115 m*M* NaCl, 2.5 m*M* KCl, 2 m*M* $CaCl_2$, and 10 m*M* HEPES, pH 7.6), 1% (v/v) fetal bovine serum (Life Technologie).

Neurotrophin Application and Microscopy

Embryonic spinal neurons from 1-day-old *Xenopus* cultures were used for these experiments. Human recombinant brain-derived neurotrophic factor (BDNF) was generously provided by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY) and stored at −85°C in small aliquots at a concentration of 10 mg/mL. Working stock solutions of BDNF at 100 μg/mL were prepared and used within 1 week. BDNF at a working concentration of 50 ng/mL was prepared in culture medium before each experiment and was applied to the culture by rapid perfusion. *Xenopus* cultures grown on a No.1 glass coverslip were mounted on an open microscopy chamber using silicon vacuum grease (Dow Corning, Midland, MI) and visualized on a Nikon Diaphot 300 inverted microscope equipped with differential interference contrast (DIC) optics. An imaging system consisting of an Argus-20 image processor (Hamamatsu Photonics, Inc., Bridgewater, NJ) and an integral CCD camera (C2400-75i, Hamamatsu) was used for video imaging and real-time contrast enhancement. Typically, the video images were background-subtracted, averaged over four video frames, and contrast-enhanced in real time. Although real-time video recording and time-lapse recording (at a standard rate of one frame every 5 s) were routinely performed (Ming et al., 1997), in this study we mostly acquired digitized still frames using the Argus-20

and a personal computer at a rate of one frame every minute for quantitative analysis. For each experiment, 5-min and 10-min recordings before and after the addition of BDNF were performed, respectively. For neurons treated with cytochalasin D (Sigma Chemicals, St. Louis, MO), nocodazole (CalBiochem, La Jolla, CA), or taxol (Molecular Probes, Eugene, OR), they were monitored during the pretreatment period to determine that these drugs exerted no drastic effects on neuronal morphology.

Double Fluorescent Labeling of Actin Filaments and Microtubules

Xenopus neurons at various times before and after the addition of BDNF were rapidly fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in a cacodylate buffer (0.1 *M* sodium cacodylate, 0.1 *M* sucrose, pH 7.4) for 30 min at room temperature. After three washes with Ringer's solution, the cells were permeabilized with 0.1% Triton X-100 in Ringer's solution for 10 min and incubated with 2% normal goat serum in Ringer's solution to block any nonspecific protein binding sites. Microtubules were labeled by incubating the permeabilized cells with a rat monoclonal antibody against β-tubulin (Bioproducts for Science, Indianapolis, IN) overnight at 4°C in a moisture chamber. The cells were then rinsed three times with Ringer's solution and incubated with a rabbit anti-rat IgG second antibody conjugated with FITC (Sigma) for 1 h at room temperature. After three extensive washes with Ringer's solution, the actin filaments were then labeled by incubating the cells with 1% rhodamine-conjugated phalloidin (Molecular Probes) for 10 min. The cells were examined immediately under a Nikon Diaphot 300 inverted microscope equipped with fluorescence attachment. Images of actin and microtubules were taken using the CCD camera with the on-chip integration feature turned on. Typically, an integration of 5–10 video frames was used to obtain high-quality fluorescent images of the actin and microtubule networks.

Image Analysis

Quantitative analysis of digitally recorded images was done using ImageTool for Windows 95, a shareware developed at the University of Texas Health Science Center, San Antonio, TX and available from the Internet by anonymous file transfer protocol from ftp://maxrad6.uthscsa.edu. Before the analysis, each image was contrast-enhanced using the contrast and brightness adjustment functions available in the program. Collateral lamellipodia were defined as the thin membrane sheets containing no intracellular organelles protruding from the organelle-rich neurite shaft. The microspikes were defined as thin processes extending from the side of the main process with a diameter of about 0.2 μm. The lamellipodial protrusion was quantified by measuring the area and the width of each lamellipodium along the neurite processes before and after the onset of BDNF; for microspikes, the number and length of microspikes along each process were measured. Some neurons did exhibit a small number of lamellipodia and microspikes before the application of BDNF and they were measured and included. All data are directly presented as the averages at different time points before and during BDNF treatment.

Quantitative analysis of microtubules in BDNF-induced lamellipodia was done by using the corresponding fluorescent images of actin networks to define the lamellipodial regions. The number of microtubules in BDNF-induced lamellipodia were measured and normalized against the width of lamellipodial protrusion (along the neurite processes) and was presented as number of MTs per 100 μm lamellipodial width. To quantify how deeply the MTs extended into the lamellipodia, we defined the *extent of MT penetration* as the ratio (in percent) between the length of the MT that extended into the lamellipodium and the length of the lamellipodium. Here, the length of the lamellipodium refers to the distance between the leading edge and the neurite shaft at the particular MT site. The higher the percentage, the closer the microtubule is to the leading edge of the lamellipodium.

RESULTS

Collateral Membrane Protrusions Induced by BDNF

Embryonic *Xenopus* spinal neurons from 24-h cultures were used for these experiments. After 24 h in culture, most *Xenopus* spinal neurons have extended considerable length of neurite processes, of which some have branched [Fig. 1(a)]. At the tip of each branch were growth cones that, unlike those from 6-h cultures, exhibit little lamellipodial protrusion and only a few filopodia, indicating the reduced motility of these growth cones (Ming et al., 1997). For most of the 24-h neurons, there was essentially no lamellipodial protrusion but a small number of lateral filopodium-like microspikes observed along the neurite shaft [arrowheads in Fig. 1(a)]. These microspikes were characterized as thin processes with an average diameter of 0.1–0.2 μm and containing no intracellular organelles detectable under high-resolution video-enhanced DIC microscopy. Acute application of 50 ng/mL BDNF induced rapid and extensive protrusion of filopodium-like microspikes [arrowheads in Fig. 1(b)] and lamellipodia [arrows in Fig. 1(b)] along the neurite shaft as well as at the growth cone. Using time-lapse microscopy, we found that BDNF-induced lamellipodia were characteristically similar to those found in many other motile cells and nerve growth cones as they exhibited both rapid protrusive activity and rearward membrane ruffling. In addition, BDNF-induced lamellipodia appeared to be quite dynamic and mobile, as they were capable of changing their morphology rapidly [Fig. 1(c)]. Most of the lamelli-

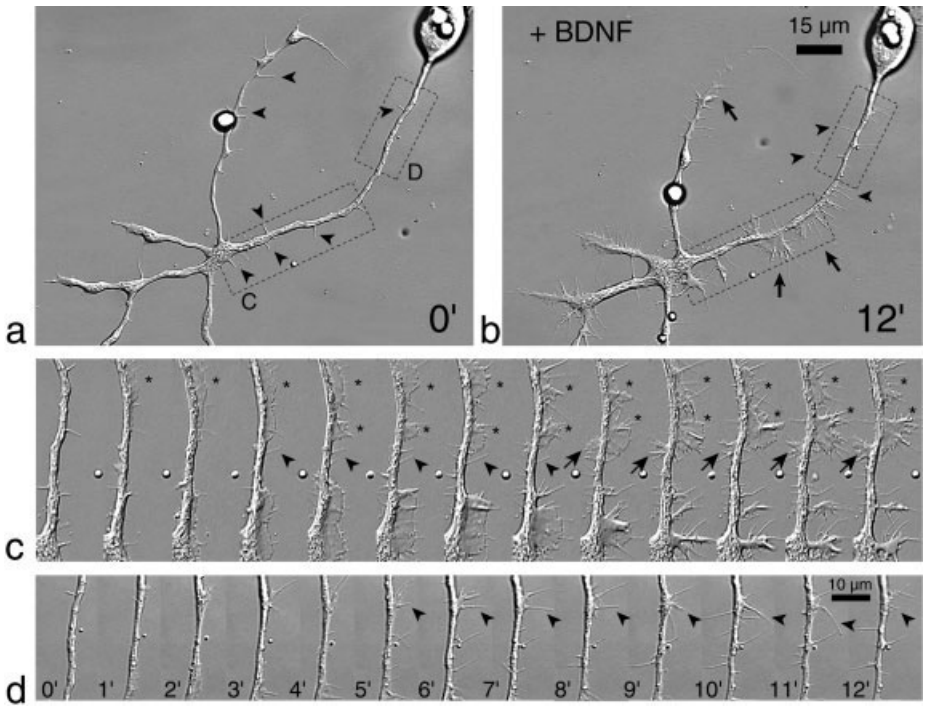

Figure 1 Extensive membrane protrusions induced by BDNF. DIC images of an embryonic *Xenopus* spinal neuron in culture before (a) and at 12 min after (b) the addition of 50 ng/mL BDNF. Both lamellipodia (arrows) and microspikes (arrowheads) were induced by BDNF. To illustrate the dynamic process of BDNF-induced membrane protrusions, DIC time-lapse sequences of two subregions of the neuron [outlined by dashed rectangles in (a) and (b)] were shown in (c) and (d). (c) BDNF induced rapid protrusion of lamellipodia and microspikes. Arrowheads indicate microspikes elicited directly from the neurite shaft. Asterisks indicate a lamellipodium that underwent rapid expansion then broke into two pieces. Although most lamellipodia appeared within the first 4 min after BDNF addition, a new lamellipodium appeared at a later stage (arrows). (d) BDNF-induced microspikes without lamellipodia. Arrowheads point to a microspike undergoing rapid extension, retraction, lateral swinging, and bending. Numbers represent the time (minutes) after the addition of BDNF. Scale bar = 10 μm.

podia were initiated within 2–3 min after the addition of BDNF and underwent rapid protrusion in the next few minutes. Although protruding outwards in a perpendicular direction to the neurite shaft, some lamellipodia also expanded along the neurite and then, sometimes, broke into pieces [asterisks in Fig. 1(c)]. Whereas some lamellipodia appeared to cease their protrusive activity and shrink in size at the end of the observation period, new lamellipodial protrusion was found to appear at later times [arrows in Fig. 1(c)]. To quantitatively analyze the dynamic features of BDNF-induced lamellipodia, we measured the size (area) of individual lamellipodium at various times after the onset of BDNF application. As depicted in Figure 2(a) and (b), most lamellipodia rapidly protruded and grew in size within the initial 2–5 min after the addition of BDNF and persisted their dynamic activities throughout the entire observation period. Prolonged time-lapse recordings showed that most BDNF-induced lamellipodial activity persisted over a period of 20–30 min and then gradually ceased (Ming et al., 1997).

Acute application of BDNF also induced lateral projection of microspikes from the neurite shaft, mostly oriented perpendicular to the shaft (arrowheads in Fig. 1). There were two types of microspikes characterized by their origins: one group of microspikes was part of lamellipodial protrusion [Fig. 1(c)] and the other was directly induced along the neurite shaft [Fig. 1(d)]. Quantitative measurement of the number of microspikes along the neurite segments shown in Figure 1(c) and (d) demonstrated that new microspikes were induced rapidly after BDNF addition in a time course similar to that of lamellipodial protrusion [Fig. 2(c)]. Moreover, the presence of lamellipodial protrusion appeared to increase the production of microspikes [Fig. 1(c), compared to Fig. 1(d)]. This is apparent at the late stage of lamellipodial protrusion at which additional numbers of micro-

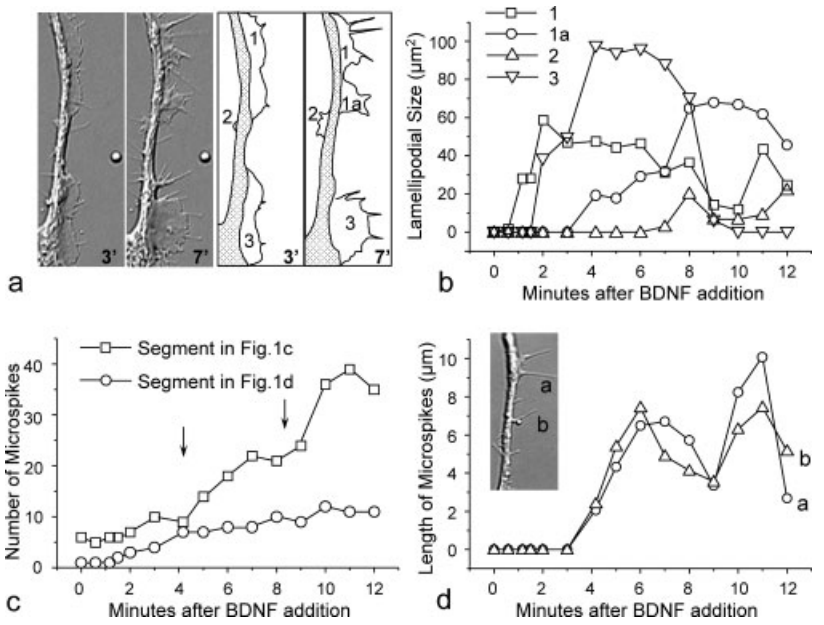

Figure 2 Quantitative analysis of membrane protrusions induced by BDNF. Lamellipodial protrusion in subregion C of the neuron shown in Figure 1 was quantified by measuring the area of each lamellipodium at various times after BDNF addition. (a) Due to the dynamic nature of these lamellipodia, each lamellipodium was outlined and labeled. Lamellipodium #1a was derived from lamellipodia #1 at the late stage of protrusion. (b) Dynamics of BDNF-induced lamellipodia as indicated by the size (area) of each lamellipodium at various times before and after the onset of BDNF application. (c) Total numbers of microspikes in subregions of the neuron outlined in Figure 1 were determined. More microspikes were elicited in subregion C where lamellipodial protrusion was observed. Arrows indicate the two step increases of the number of microspikes at the late stages of protrusion. (d) The dynamics of two representative microspikes (labeled as a and b) as demonstrated by their lengths at various times before and after BDNF addition. Both spikes underwent two cycles of rapid extension and retraction with the 12-min period.

spikes were observed [arrows in Fig. 2(c)]. Similar to filopodia seen at motile growth cones, BDNF-induced microspikes appeared quite dynamic and exhibited a number of movements including rapid extension and retraction, lateral swinging, and bending [arrowheads in Fig. 1(d)]. Quantitative analysis of the length of two representative microspikes [Fig. 2(d)] demonstrated their dynamic characters as well as the cycles of extension and retraction that each microspike experienced during the observation period.

Actin Filaments and Microtubules in BDNF-Induced Lamellipodia

It is believed that the core structure of lamellipodia is the actin cytoskeleton assembled into a meshwork (Small, 1994). Using fluorescence staining method, we labeled both the actin and microtubule cytoskeleton in *Xenopus* neurons fixed before and at various times after the onset of BDNF application. Images of actin filaments (AFs) and MTs of same regions of each neuron are presented in pairs in Figure 3 (left panel: AFs; right panel: MTs). In the absence of BDNF treatment, actin filaments were concentrated mainly in the growth cone, whereas a thin layer of cortical actin meshwork was observed beneath the plasma membrane in the neurite shaft [Fig. 3(a)]. Microtubules, on the other hand, were distributed in a pattern complementary to the actin cytoskeleton: densely packed parallel bundles were seen in the neurite shaft and mostly terminated at the C-region of the growth cone [Fig. 3(a), (see also Smith, 1988; Zheng et al., 1996)]. Very few MTs were observed in the filopodia and lamellipodia at the growth cone. Within minutes after BDNF addition, extensive membrane protrusion was observed at multiple sites along the neurite shaft as well as at the growth cone [Fig. 3(b)–(f)]. This was clearly indicated by the accumulation of actin filaments at each protrusion site along the neurite shaft at the early stage of protrusion [Fig. 3(b)]. With the lamellipodia protruding further, the actin distribution of these lamellipodia became more

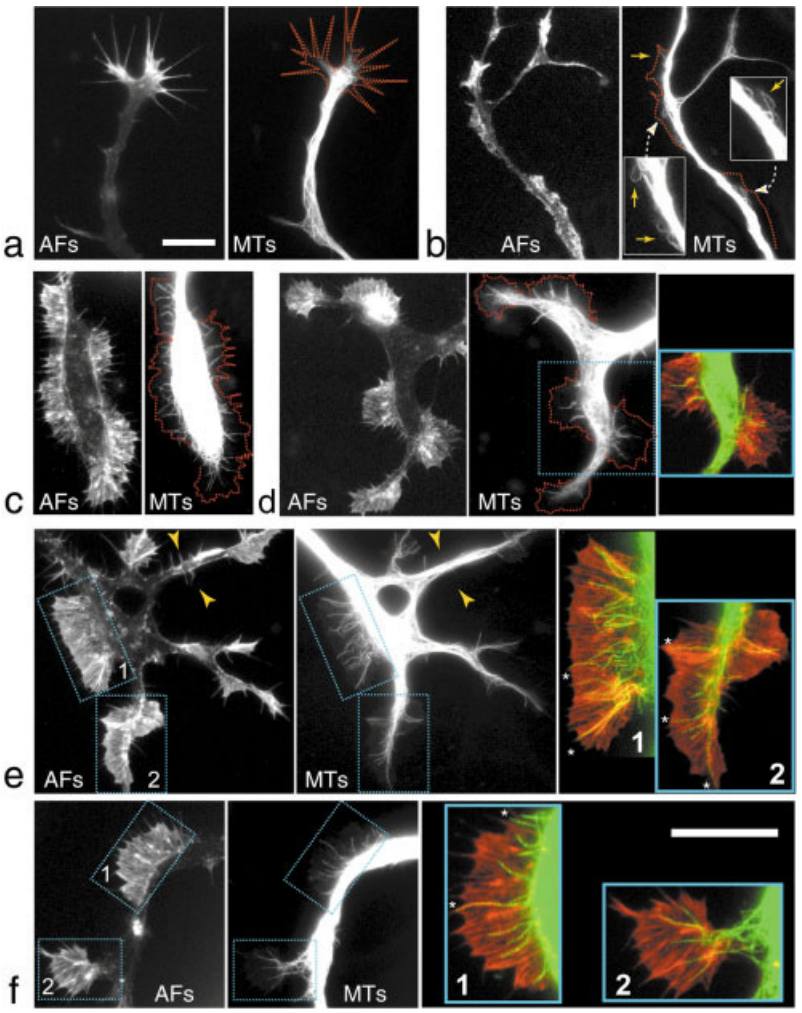

Figure 3 Distributions of MTs and the actin filaments (AFs) in lamellipodia and microspikes induced by BDNF. Cultured *Xenopus* neurons were fixed and double labeled for actin filaments and MTs before (a) and at 2 min (b), 4 min [(c) and (d)], 8 min (e), and 10 min (f) after the addition of BDNF. Images of actin filaments and MTs are presented in pairs for each neuron (left panel: actin filaments; right panel: MTs). For clarity, lamellipodial regions in MT images were outlined by red dotted lines. Color images are superimposed images of MTs and actin filaments of the same regions outlined by dotted rectangles; They were enlarged to twice their original sizes to provide more detail on the distribution of MTs and actin filaments. Scale bars: 15 μm. In (b), MT loops in lamellipodia are indicated by arrows and the magnified insets (2$\times$ the original). Arrowheads in (e) indicate microspikes that contain only actin filaments but not MTs. Asterisks in the insets indicate MTs that reached the leading edge of lamellipodia.

typical of lamellipodia seen in other cell types, namely, actin filaments formed a crisscross network interspersed by larger bundles (ribs) in the lamellipodia [Fig. 3(c)–(f)]. Some of these actin ribs extended out of the lamellipodia to form the microspikes. Such distribution of actin filaments was observed in lamellipodia induced along the neurite shaft as well as at the growth cone [Fig. 3(c)–(e)], suggesting the same cellular mechanisms underlie BDNF-induced lamellipodial protrusion at different subcellular locations.

The distribution of the actin cytoskeleton in BDNF-induced lamellipodia resembled closely that of motile growth cones and other motile cells. Strikingly, however, BDNF-induced lamellipodia were found to contain microtubules. The appearance of MTs in lamellipodia was observed as early as 2 min after BDNF addition [Fig. 3(b)], the earliest stage for the clear identification of the lamellipodial protrusion. We first observed "loops" of MTs on the side of densely packed MT bundles in the neurite shaft [ar-

Table 1 Characteristics of MTs in BDNF-Induced Lamellipodia[a]

Time (min) after BDNF Addition	Extent of MT Penetration in Lamellipodia[b]	Number of MTs per 100 μm width of Lamellipodia	% of MTs that Reached the Leading Edge of Lamellipodia	% of MTs That Reached the Leading Edge with Buckling Morphology	Angles of MTs in Lamellipodia in Respect to the Neurite Shaft[c]	Number of Cells Examined
0	N/A	N/A	N/A	N/A	N/A	
2	81.6 ± 2.7	21.0 ± 5.2	45.1 ± 6.8	43.8 ± 16.4	49.8 ± 4.6	36
4	77.0 ± 2.3	45.2 ± 6.0	59.2 ± 7.1	14.2 ± 5.9	84.5 ± 3.2	22
6	77.8 ± 5.7	28.7 ± 6.5	48.4 ± 11.2	25.0 ± 12.5	70.3 ± 5.7	16
8	80.5 ± 2.9	41.6 ± 7.5	60.0 ± 6.8	33.7 ± 10.4	77.0 ± 3.8	11
10	78.0 ± 4.1	28.7 ± 7.4	61.1 ± 10.1	19.0 ± 13.2	75.1 ± 5.6	10

[a] Data are presented as MEAN ± S.E.M.

[b] This is calculated by the length of the MT extended in a lamellipodium divided by the length of the lamellipodium (along the axis of growth of the leading edge). See Methods for definition.

[c] An angle of 90 degree indicates that MTs are perfectly perpendicular to the neurite shaft.

rows, also see the magnified insets in Fig. 3(b)]. These MT loops were located in the protruding lamellipodia as confirmed by the faint background as well as the corresponding actin staining (in rhodamine fluorescence channel) of the newly induced lamellipodia. At the later stages of lamellipodial protrusion, MTs appeared to straighten out and single MTs (or thin bundles) with free ends were found to extend in the lamellipodia, pointing towards the direction of the membrane protrusion [Fig. 3(c) and (d)]. The number of MTs in lamellipodia increased at 4 min after the onset of BDNF application (Table 1). At this and later time points, MTs were observed in nearly all the lamellipodia induced by BDNF, both along the neurite shaft and at the growth cone. Most of the MTs in lamellipodia had extended deeply towards the leading edge, which was quantified by the *extent of MT penetration* in lamellipodia (Table 1, see Methods for definition). Overall, MTs in lamellipodia extended over 80% of the protruding length of lamellipodia and a large percentage of the MTs (~50%) actually reached the leading edge of the lamellipodia [Table 1, see also Fig. 3(e) and (f)]. Furthermore, we observed the unique "buckling" morphology of some MTs in the lamellipodia, suggesting that these MTs experienced compressive force exerted by other cellular structures [Fig. 3(c)–(f) and Table 1]. In contrast to lamellipodia, most microspikes induced by BDNF did not contain MTs, but they did contain thick bundles of actin filaments [arrowheads in Fig. 3(e)]. For example, at 10 min after BDNF addition, only 18.2 ± 5.8% of the microspikes contained MTs ($N = 8$). For this small percentage of microspikes containing microtubules, we suspect that they might not be induced by BDNF but rather were minor branches that existed before BDNF application.

To examine the relationship between actin filaments and microtubules in the lamellipodia, we superimposed the images of actin filaments and microtubules of the same regions of the neurons containing lamellipodia (outlined by dashed rectangles) with a pixel-to-pixel accuracy [color images in Fig. 3(d)–(f)]. It was further confirmed that most of the MTs have extended quite deeply in the lamellipodia with some of them actually reaching the leading edge of the lamellipodia [asterisks in Fig. 3(e) and (f)]. When comparing the distributions of MTs and actin filaments, it is clear that MTs in the lamellipodia did not overlap with the thick actin bundles in the lamellipodia, suggesting that these two cytoskeletal structures may be involved in different aspects of BDNF-induced membrane protrusion.

Dependence of BDNF-Induced Membrane Protrusions on Cytoskeletal Structures

The appearance of actin filaments in both lamellipodia and microspikes suggest that these two types of membrane protrusions require the polymerization of actin filaments. To test it, cytochalasins were used to inhibit the polymerization of actin filaments. With the presence of 0.5 μM cytochalasin D in the culture medium, acute application of 50 ng/mL BDNF did not induce any morphologic changes in *Xenopus* neurons; no protrusion of lamellipodia or microspikes was observed upon BNDF application [Fig. 4(a)]. This result demonstrated that BDNF-induced protrusion of lamellipodia and microspikes depends on the actin cytoskeleton and polymerization of actin filaments is required for both protrusive activities.

The presence of MTs in lamellipodia induced by

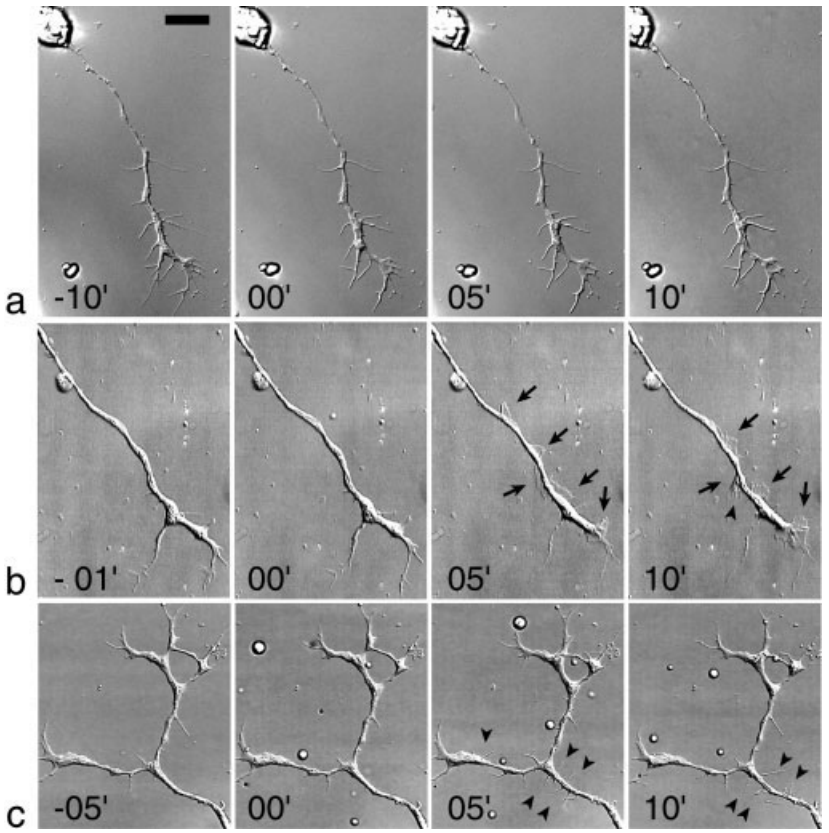

Figure 4 Dependence of BDNF-induced membrane protrusion on the cytoskeleton. (a) BDNF-induced protrusion of both lamellipodia and microspikes was completely abolished by 0.5 μM cytochalasin D. The time-lapse DIC sequence shows the neuron at various times before and after the addition of 50 ng/mL BDNF (at 00 min); 0.5 μM cytochalasin D was added 10 min prior to the addition of BDNF. Digits represent time in minutes. Scale bar = 20 μm. (b) BDNF-induced membrane protrusion was not affected by 5 μM taxol. Both lamellipodia (arrows) and microspikes (arrowheads) were observed. (c) Inhibition of MT polymerization by 20 μM nocodazole completely blocked the formation of lamellipodia induced by BDNF. However, microspikes were still induced by BDNF in the presence of nocodazole (arrowheads).

BDNF also suggests the potential involvement of MTs in lamellipodial protrusion. To test the role of MTs in BDNF-induced membrane protrusions, we examined BDNF-induced membrane protrusions in neurons exposed to drugs that either stabilize or depolymerize MTs. Application of 5 μM taxol, an MT-stabilizing drug, did not inhibit BDNF-induced formation of lamellipodia and microspikes [Fig. 4(b)]. No obvious effect of taxol on lamellipodial protrusion was observed. However, whether any of the dynamic properties of the lamellipodia (e.g., extension rate, retraction rate, etc.) was affected remains to be determined. Immunofluorescent staining showed that some MTs were still observed in BDNF-induced lamellipodia but mostly in short as well as "loop" morphology without extending deep in the lamellipodia [Fig. 5(a)]. On the other hand, when 20 μM nocodazole, a MT-disrupting drug, was applied to *Xenopus* neurons, BDNF-induced lamellipodial protrusion was largely blocked [Fig. 4(c)]. Immunostaining showed that nocodazole treatment depolymerized most of MTs [Fig. 5(b)]. However, the nocodazole treatment did not appear to damage the neuron nor did it significantly alter the morphology of the neuron. It should be emphasized that the blocking effect of nocodazole on BDNF-induced lamellipodial formation does not appear to result from nonspecific action of the drug. In the presence of nocodazole, collateral sprouting of microspikes was still induced by BDNF [arrowheads in Fig. 4(c)], indicating the specificity of nocodazole on lamellipodial protrusion. Quantitative analysis of populations of neurons clearly showed that only the formation of lamellipodia but not of microspikes induced by BDNF was blocked by nocodazole; neither the number nor the average length of microspikes induced by BDNF was affected by nocodazole (Fig.

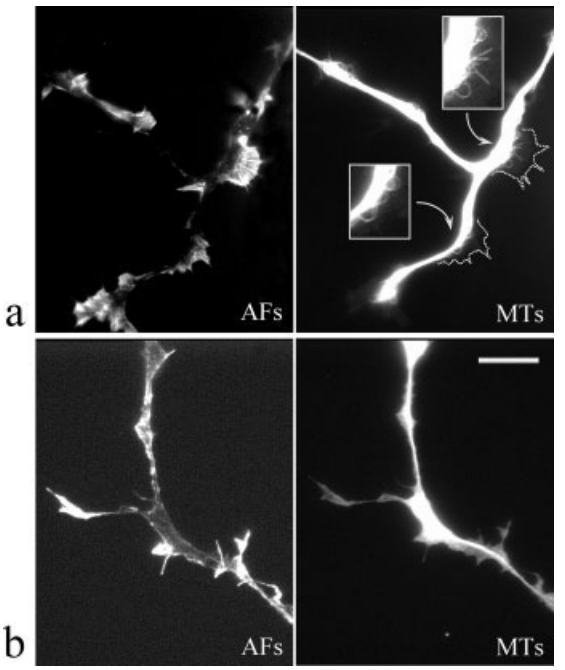

Figure 5 Distributions of MTs and actin filaments in neurons treated with taxol and nocodazole. (a) Images of actin filaments (AFs) and MTs of a taxol-treated neuron at 6 min after the addition of 50 ng/mL BDNF. Lamellipodia were observed along the neurite shaft. As in the control neurons exposing to BDNF, some MTs were observed in BDNF-induced lamellipodia but in short and "loop" morphology (see also the 2× magnified insets). For clarity, lamellipodial regions in the image of MTs were outlined by dotted lines. (b) Images of AFs and MTs of a nocodazole-treated neuron at 6 min after the addition of BDNF. Although some actin accumulation was observed along the neurite shaft, no lamellipodial protrusion was found. Most of MTs were depolymerized by nocodazole treatment. Scale bar = 15 μm.

6). Furthermore, similar inhibition of BDNF-induced lamellipodial protrusion was also observed with vinblastine, another MT-disrupting drug (data not shown), thus supporting the result of specific blockade of lamellipodial protrusion by nocodazole. Taken together with the observation from the immunofluorescent study, these results demonstrated that both lamellipodia and microspikes are actin-based membrane protrusions elicited by BDNF; lamellipodial protrusion, however, also requires active participation of MTs.

DISCUSSION

Neurotrophins have been shown to be involved in axonal and dendritic sprouting and branching during neuronal development (Diamond et al., 1992; Cohen-Cory and Fraser, 1995; McAllister et al., 1996). The effective BDNF concentration used here to induce membrane protrusions is very similar to that previously shown in axonal and dendritic sprouting and branching. The rapid protrusion of filopodia and la-

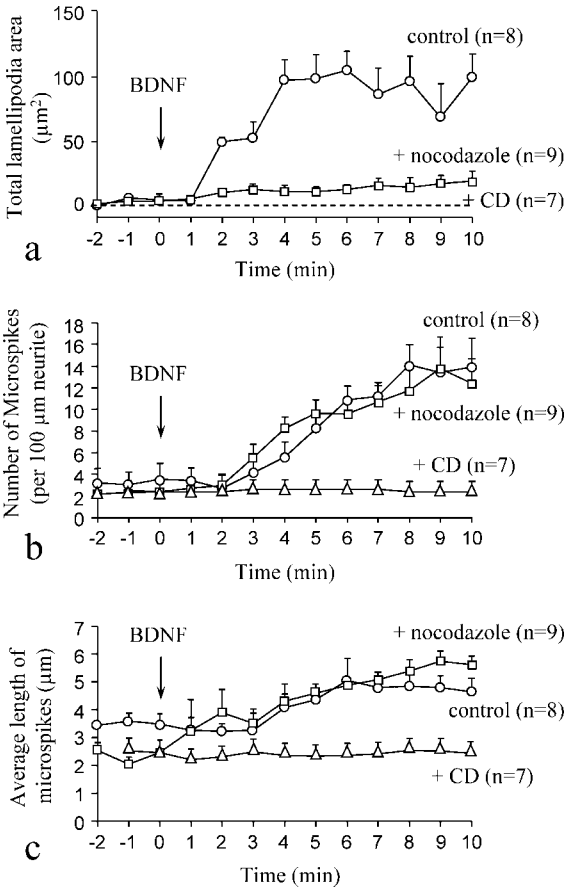

Figure 6 Quantitative analysis of the dependence of lamellipodia and microspikes on the actin and microtubule cytoskeleton. (a) Lamellipodial protrusion induce by BDNF was blocked by either 0.5 μM cytochalasin D (CD) or 20 μM nocodazole. Dashed line represents the results from CD that completely blocked BDNF-induced lamellipodial protrusion. Nocodazole also abolished the lamellipodial protrusion induced by BDNF. (b) Analysis of the average number of microspikes induced by BDNF per 100 μm neurite length showed that the induction of microspikes was blocked by CD but not by nocodazole. The number of microspikes induced by BDNF in the presence of nocodazole showed no difference from that of control (BDNF only). It should be emphasized, however, that a small number of microspikes existed before BDNF application and was not eliminated by either CD or nocodazole treatment. (c) Analysis of the average length of microspikes also showed that nocodazole did not affect the length of microspikes induced by BDNF. However, CD reduced the length of microspikes that existed before the application of BDNF.

mellipodia observed in this study could well be the early event that leads to the collateral sprouting of neurite branches (Yu et al., 1994; Gallo and Letourneau, 1998). Furthermore, rapid formation of dendritic filopodia has been suggested as a mechanism involved in the plasticity of neuronal connections (Ziv and Smith, 1996). The extensive protrusion of microspikes and lamellipodia induced by BDNF, if occurring at the synaptic terminals, could contribute to the modulation of synaptic connectivity by neurotrophins via initiating new contacts between presynaptic and postsynaptic cells. In this study, we have analyzed in detail the extensive membrane protrusions induced by BDNF. In particular, we have identified and characterized two distinct types of membrane protrusions induced by BDNF: filopodium-like microspikes and lamellipodia. We then show that different cytoskeletal components are involved in these two types of membrane protrusions. Although BDNF-induced microspikes depend solely on the actin cytoskeleton, BDNF-induced lamellipodial protrusion depends on both the actin and microtubule cytoskeleton. These results thus provide important insights into the cellular mechanisms underlying the morphologic responses of neurons to extrinsic factors.

We have shown that BDNF-induced membrane protrusions involve the high-affinity Trk receptors (Ming et al., 1997). Although the exact downstream intracellular signaling events have not been determined, it is clear that both BDNF-induced microspikes and lamellipodia involve the reorganization of the actin cytoskeleton. Previous studies on growth factor-induced membrane ruffling in non-neuronal cells suggested that phosphatidylinosital-3 (PI-3) kinase and the Rho family of small GTPases are directly involved in the actin rearrangement required for membrane ruffling (Ridley et al., 1992; Wennstrom et al., 1994; Kotani et al., 1995; Parker, 1995). Activation of Trk receptor tyrosine kinases by neurotrophins also activates PI-3 kinases (Ohmichi et al., 1992; for review, see Kaplan and Stephens, 1994). Our preliminary study found that both PI-3 kinases and the Rho-family small GTPases were involved in BDNF-induced membrane protrusions. For example, application of 15 μM LY-294002, a specific inhibitor for PI-3 kinases (Vlahos et al., 1994), largely attenuated BDNF-induced lamellipodial protrusion. The total lamellipodia area per 100 μm neurite length at 10 min after the addition of BDNF was reduced from 63 $\pm$ 11 μm^2 ($N = 29$) to 6 $\pm$ 3 μm^2 ($N = 34$) by the presence of LY-294002. Moreover, application of 100 pg/mL toxin B (*Clostridium difficile*; Just et al., 1995) that specifically inhibits the Rho-family GTPases attenuated BDNF-induced lamellipodia from 73 $\pm$ 16 μm^2 ($N = 28$) to 10 $\pm$ 5 μm^2 ($N = 36$). Similarly, the number of microspikes induced by BDNF was also largely reduced by LY-294002 and toxin B (data not shown). These results indicate the PI-3 kinase and Rho GTPases signaling pathway in BDNF-induced membrane protrusions.

The major finding of this study is the involvement of different cytoskeletal components in microspikes and lamellipodia induced by BDNF. Lamellipodia have long been viewed as the actin-based dynamic structure associated with cell motility (Smith, 1988; Heath and Holifield, 1991; Okabe and Hirokawa, 1991; Lin et al., 1994; Small, 1994). The involvement of microtubules in actin-based cellular activities such as lamellipodial protrusion is less documented. Our results now provide the evidence that the actin and microtubule cytoskeletal systems work cooperatively in the development of lamellipodial protrusion induced by BDNF. The presence of MTs in lamellipodia from the very beginning of protrusion and the blockade of lamellipodia formation by MT-disrupting drugs suggest an active role for MTs in lamellipodial protrusion. These results are consistent with the recent finding that interactions between MTs and the actin cytoskeleton are required for axon branching (Dent and Kalil, 2001). It is unclear on the exact role of MTs in lamellipodial initiation and protrusion. In a small number of neurons exposed to nocodazole, acute application of BDNF was found to elicit some membrane ruffling that resembles the early lamellipodial protrusion observed in cells without nocodazole treatment (note the nocodazole curve in Fig. 6). Most of the ruffling, however, existed only briefly and never fully developed into lamellipodia with the distinct morphology. This result, together with the observations of the complete blockade of membrane protrusion by cytochalasins and the persistent protrusion of microspikes in the presence of nocodazole induced by BDNF, suggests that the actin cytoskeleton is the primary target of BDNF-signaling for inducing both types of membrane protrusion; the further development of lamellipodia, however, requires the active participation of MTs.

Microtubules in the axonal shaft are typically closely packed and arranged axially with uniform polarity (Hirokawa, 1991). They are rather insensitive to microtubule depolymerizing drugs, and therefore presumably not very dynamic, in a sharp contrast to the microtubule behavior in the growth cone. The appearance of "loop" morphology of MTs at the protrusion sites at the early protrusive stage followed by single MTs with free ends indicated that debundling, fragmentation, and polymerization of MTs occurred during BDNF-induced lamellipodial protrusion along

the neurite shaft, which is consistent with MT behaviors during axon branching (Yu et al., 1994; Dent and Kalil, 2001). The observation of short MTs and "loops" in BDNF-induced lamellipodia in the presence of taxol suggests that the deep extension of MTs into the lamellipodia is not necessary for BDNF-induced lamellipodial protrusion. Rather, the local debundling and fragmentation of MTs at the protrusion sites of the neurite process, possibly together with polymerization, may provide a mechanism for the development of lamellipodia. It is likely that interactions between dynamic MTs and actin filaments involve coordinated polymerization and depolymerization of both cytoskeletal systems. Such interactions may represent a common mechanism underlying many types of cell motility (Dent and Kalil, 2001; Salmon et al., 2002; Schaefer et al., 2002). Although the exact nature of interactions between MTs and the actin cytoskeleton remains to be further determined, one potential candidate is the Rho-family of small GTPases that regulate the actin cytoskeletal architecture and dynamics (Hall, 1998). Recent studies have demonstrated the direct involvement of the Rho-family GTPases in crosstalk between MTs and the actin cytoskeleton (Waterman-Storer and Salmon, 1999; Krendel et al., 2002; Fukata et al., 2002). Specifically, Rac1, a member of the Rho family, was shown to be activated by MT growth to induce lamellipodial protrusion. Using Toxin B to inhibit the Rho-family of small GTPases, we have shown that BDNF-induced lamellipodial protrusion was largely attenuated. It is thus possible that Rho GTPases could mediate the interaction and coordination between these two cytoskeletal systems for BDNF-induced lamellipodial protrusion. Furthermore, the formation of new lamellipodia along the neurite shaft likely requires new membrane to be added to the surface through exocytosis. Although little exocytosis was observed along the neurite shaft of *Xenopus* neurons under normal conditions, local destabilization/debundling/fragmentation of MTs in the middle region of the shaft was found necessary and sufficient to induce exocytosis locally (Zakharenko and Popov, 1998). It is possible that the local debundling and fragmentation of MTs along the neurite shaft may allow the local addition of new membrane to the surface for the formation of lamellipodia induced by BDNF (Fig. 7). One could thus speculate that the selective debundling, fragmentation, and polymerization of MTs, through interacting with the actin cytoskeleton and promoting membrane addition, may specifically drive the lamellipodial protrusion at particular locations along the neurite shaft in response to extracellular factors such as BDNF (Fig. 7).

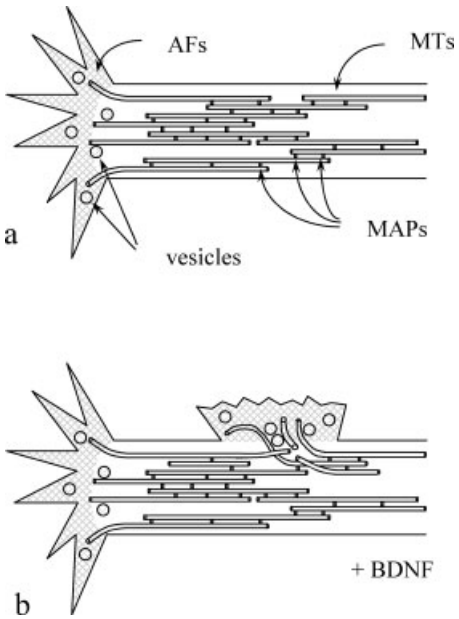

Figure 7 Schematic diagram illustrating the model in which local debundling, fragmentation, and/or polymerization of MTs along neurite shaft contributes to the formation of lamellipodial protrusion induced by BDNF. (a) Under the normal conditions, MTs in the neurite shaft are highly bundled (crosslinked) by microtubule-associated proteins (MAPs). Membrane exocytosis is mostly restricted at the growth cone where some MTs are splayed with free ends. (b) Upon BDNF application, the actin-based membrane ruffling is elicited. At the same time, some MTs at the ruffling sites are induced to undergo debundling, fragmentation, and polymerization, resulting in single MTs with free ends. These dynamic MTs may thus interact with the actin cytoskeleton to induce coordinated polymerization to drive the actin-based protrusive activity. Moreover, these dynamic MTs could also allow the addition of new membrane to the cell surface through local exocytosis, leading to the further development of lamellipodia.

The observed active involvement of MTs in BDNF-induced protrusion of lateral lamellipodia along the neurite processes may have more general implications in many cellular events involving lamellipodial activity, in particular, in growth cone pathfinding. It has been observed that single microtubules (or bundles) occasionally enter the lamellipodial region of the growth cones during pathfinding (Letourneau, 1983; Gordon-Weeks, 1991; Tanaka and Kirschner, 1991). Recent studies indicate that selective advance of MTs into specific regions of the growth cone may play a key role in growth cone steering in response to extracellular cues (Lin and Forscher, 1993; Bentley and TP, 1994; Tanaka and Kirschner, 1995). It is unclear whether MTs play an active role to steer the growth cones in response to

extracellular signals or a supporting role to consolidate the activities of the actin cytoskeleton at the growth cone. Challacodmbe et al. (Challacombe et al., 1997) showed that inhibition of MT assembly/disassembly dynamics reduced lamellipodial area and blocked the repulsion of the growth cone by an inhibitory cue, implicating an active role for MTs in the actin-based growth cone motility and pathfinding. We have recently shown that direct local modification of MT dynamics at the growth cone was sufficient to induce turning responses, demonstrating an instructive role for MTs in growth cone steering (Buck and Zheng, 2002). It is likely that microtubules and the actin cytoskeleton work together to steer nerve growth cones in response to extracellular cues. External guidance cues could initiate signaling cascades that target the actin cytoskeleton, which in turn, regulates the behaviors of MTs. On the other hand, recent studies have shown that the dynamic instability of MTs is regulated by a large number of cellular factors, including the structural MT-associated proteins (MAPs). Furthermore, MT-affinity regulating kinases (MARKs) are novel serine/threonine kinases that phosphorylate the tubulin domain of MAPs and therefore cause their detachment from MTs and increased MT dynamics (Drewes et al., 1998). Therefore, extracellular molecules may activate the signaling pathway that acts directly on MTs through MAPs and MARKs, leading to the regulation of the dynamics of individual MTs at the growth cone. Nevertheless, selective and preferential extension of MTs is required, in conjunction with the activity of the actin cytoskeleton, to produce the lamellipodial membrane protrusion in a defined direction to steer the growth cones.

REFERENCES

Bastmeyer M, Daston MM, Possel H, O'Leary DD. 1998. Collateral branch formation related to cellular structures in the axon tract during corticopontine target recognition. J Comp Neurol 392:1–18.

Bentley D, TP OC. 1994. Cytoskeletal events in growth cone steering. Curr Opin Neurobiol 4:43–48.

Berninger B, Poo MM. 1996. Fast actions of neurotrophic factors. Curr Opin Neurobiol 6:324–330.

Bray D, Hollenbeck PJ. 1988. Growth cone motility and guidance. Annu Rev Cell Biol 4:43–61.

Buck KB, Zheng JQ. 2002. Growth cone turning induced by direct local modification of microtubule dynamics. J Neurosci, in press.

Burmeister DW, Rivas RJ, Goldberg DJ. 1991. Substrate-bound factors stimulate engorgement of growth cone lamellipodia during neurite elongation. Cell Motil Cytoskelet 19:255–268.

Challacombe JF, Snow DM, Letourneau PC. 1997. Dynamic microtubule ends are required for growth cone turning to avoid an inhibitory guidance cue. J Neurosci 17:3085–3095.

Cohen-Cory S, Fraser SE. 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature 378:192–196.

Davies AM. 1994. The role of neurotrophins in the developing nervous system. J Neurobiol 25:1334–1348.

Dent EW, Kalil K. 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. J Neurosci 21:9757–9769.

Diamond J, Holmes M, Coughlin M. 1992. Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. J Neurosci 12:454–466.

Drewes G, Ebneth A, Mandelkow EM. 1998. MAPs, MARKs and microtubule dynamics. Trends Biochem Sci 23:307–311.

Fiala JC, Feinberg M, Popov V, Harris KM. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. J Neurosci 18:8900–8911.

Fukata M, Watanabe T, Noritake J, Nakagame J, Yamaga M, Kuroda S, Matsuura Y, Iwamatsu A, Perez F, Kaibuchi K. 2002. Rac1 and cdc42 capture microtubles through IQGAP1 and CLIP-170. Cell 109:873–885.

Gallo G, Letourneau PC. 1998. Localized sources of neurotrophins initiate axon collateral sprouting. J Neurosci 18:5403–5414.

Goldberg DJ, Burmeister DW. 1989. Looking into growth cones. Trends Neurosci 12:503–506.

Goldberg DJ, Burmeister DW. 1992. Microtubule-based filopodium-like protrusions form after axotomy. J Neurosci 12:4800–4807.

Gordon-Weeks PR. 1991. Evidence for microtubule capture by filopodial actin filaments in growth cones. Neuroreport 2:573–576.

Goslin K, Birgbauer E, Banker G, Solomon F. 1989. The role of cytoskeleton in organizing growth cones: a microfilament-associated growth cone component depends upon microtubules for its localization. J of Cell Biol 109:1621–1631.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279:509–514.

Heath J, Holifield B. 1991. Cell locomotion. Actin alone in lamellipodia. Nature 352:107–108.

Hirokawa N. 1991. Molecular architecture and dynamics of the neuronal cytoskeleton. In: Burgoyne RD, editor. Burgoyne RDs. The neuronal cytoskeleton. New York: Wiley-Liss. p 5–74.

Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. 1995. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature 375:500–503.

Kaplan DR, Stephens RM. 1994. Neurotrophin signal transduction by the Trk receptor. J Neurobiol 25:1404–1417.

Kotani K, Hara K, Kotani K, Yonezawa K, Kasuga M. 1995. Phosphoinositide 3-kinase as an upstream regulator of the small GTP-binding protein Rac in the insulin signaling of membrane ruffling. Biochem Biophys Res Commun 208:985–990.

Krendel M, Zenke FT, Bokoch GM. 2002. Nucleotide ex-

change factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. Nat Cell Biol 4:294–301.

Letourneau PC. 1983. Differences in the organization of actin in the growth cones compared with the neurites of cultured neurons from chick embryos. J Cell Biol 97: 963–973.

Lin CH, Forscher P. 1993. Cytoskeletal remodeling during growth cone-target interactions. J Cell Biol 121:1369–1383.

Lin CH, Forscher P. 1995. Growth cone advance is inversely proportional to retrograde F-actin flow. Neuron 14:763–771.

Lin CH, Thompson CA, Forscher P. 1994. Cytoskeletal reorganization underlying growth cone motility. Curr Opin Neurobiol 4:640–647.

Lockerbie RO. 1987. The neuronal growth cone: a review of its locomotory, navigational and target recognition capabilities. Neuroscience 20:719–729.

Lohof AM, Ip NY, Poo MM. 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363:350–353.

Maletic-Savatic M, Malinow R, Svoboda K. 1999. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity [see comments]. Science 283:1923–1927.

McAllister AK, Katz LC, Lo DC. 1996. Neurotrophin regulation of cortical dendritic growth requires activity. Neuron 17:1057–1064.

Ming GL, Lohof AM, Zheng JQ. 1997. Acute morphogenic and chemotropic effects of neurotrophins on cultured embryonic *Xenopus* spinal neurons. J Neurosci 17:7860–7871.

Mitchison T, Kirschner M. 1988. Cytoskeletal dynamics and nerve growth. Neuron 1:761–772.

Morfini G, DiTella MC, Feiguin F, Carri N, Caceres A. 1994. Neurotrophin-3 enhances neurite outgrowth in cultured hippocampal pyramidal neurons. J Neurosci Res 39:219–232.

Nieuwkoop PD, Faber J. 1967. Normal table of *Xenopus laevis*. Amsterdam: Elsevier.

Ohmichi M, Decker SJ, Saltiel AR. 1992. Activation of phosphatidylinositol-3 kinase by nerve growth factor involves indirect coupling of the trk proto-oncogene with src homology 2 domains. Neuron 9:769–777.

Okabe S, Hirokawa N. 1991. Actin dynamics in growth cones. J Neurosci 11:1918–1929.

Parker PJ. 1995. Intracellular signalling. PI 3-kinase puts GTP on the Rac. Curr Biol 5:577–579.

Patel MN, McNamara JO. 1995. Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. Neuroscience 69:763–770.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410.

Robbins N, Polak J. 1988. Filopodia, lamellipodia and retractions at mouse neuromuscular junctions. J Neurocytol 17:545–561.

Salmon WC, Adams MC, Waterman-Storer CM. 2002. Dual-wavelength fluorescent speckle microscopy reveals coupling of microtubule and actin movements in migrating cells. J Cell Biol 158:31–37.

Schaefer AW, Kabir N, Forscher P. 2002. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. J Cell Biol 158:139–152.

Small JV. 1994. Lamellipodia architecture: actin filament turnover and the lateral flow of actin filaments during motility. Semin Cell Biol 5:157–163.

Smith SJ. 1988. Neuronal cytomechanics: the actin-based motility of growth cones. Science 242:708–715.

Spitzer NC, Lamborghini JE. 1976. The development of the action potential mechanism of amphibian neurons isolated in culture. Proc Natl Acad Sci USA 73:1641–1645.

Tabti N, Poo MM. 1990. Culturing spinal cord neurons and muscle cells from *Xenopus* embryos. In: Banker G, Goslin K, Banker G, Goslin Ks, editors. Culuturing nerve cells. Cambridge: MIT, p 137–154.

Tanaka E, Kirschner MW. 1995. The role of microtubules in growth cone turning at substrate boundaries. J Cell Biol 128:127–137.

Tanaka EM, Kirschner MW. 1991. Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115:345–363.

Vega LR, Solomon F. 1997. Microtubule function in morphological differentiation: growth zones and growth cones. Cell 89:825–828.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248.

Wang Q, Zheng JQ. 1998. Cyclic AMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. J Neurosci 18:4973–4984.

Waterman-Storer CM, Salmon ED. 1997. Actomyosin-based retrograde flow of microtubules in the lamella of migrating epithelial cells influences microtubule dynamic instability and turnover and is associated with microtubule breakage and treadmilling. J Cell Biol 139:417–434.

Waterman-Storer CM, Salmon E. 1999. Positive feedback interactions between microtubule and actin dynamics during cell motility. Curr Opin Cell Biol 11:61–67.

Wennstrom S, Hawkins P, Cooke F, Hara K, Yonezawa K, Kasuga M, Jackson T, Claesson-Welsh L, Stephens L. 1994. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. Curr Biol 4:385–393.

Yu W, Ahmad FJ, Baas PW. 1994. Microtubule fragmentation and partitioning in the axon during collateral branch formation. J Neurosci 14:5872–5884.

Zakharenko S, Popov S. 1998. Dynamics of axonal microtubules regulate the topology of new membrane insertion into the growing neurites. J Cell Biol 143:1077–1086.

Zheng JQ, Wan JJ, Poo MM. 1996. Essential role of filopodia in chemotropic turning of nerve growth cone induced by a glutamate gradient. J Neurosci 16:1140–1149.

Ziv NE, Smith SJ. 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17:91–102.