

cAMP-Mediated Regulation of Neurotrophin-Induced Collapse of Nerve Growth Cones

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Neurotrophins are known to promote the survival, differentiation, and neurite outgrowth of developing neurons. Here we report that acutely applied brain-derived neurotrophic factor (BDNF) induces rapid growth cone collapse and neurite retraction of embryonic *Xenopus* spinal neurons in culture. The collapsing effect of BDNF depends on the activation of Trk receptor tyrosine kinase, requires an influx of extracellular Ca^{2+} , and is regulated by cAMP-dependent activity. Elevation of intracellular cAMP levels ($[cAMP]_i$) by forskolin or (S_p)-cAMP completely blocked the collapsing effect, whereas inhibition of protein kinase A (PKA) by (R_p)-cAMP potentiated the collapsing action. BDNF-induced growth cone collapse was only observed in 6 hr cultures but not in 24 hr cultures. However, inhibition of PKA by (R_p)-cAMP restored the collapsing response of these "old" neurons in 24 hr cultures, suggesting that

embryonic *Xenopus* spinal neurons may upregulate their endogenous cAMP-dependent activity during development in culture, leading to the blockade of their collapsing response to BDNF. Taken together, our results suggest the presence of cross-talk between Ca^{2+} - and cAMP-signaling pathways involved in the collapsing action of neurotrophins, in which the cAMP-pathway regulates the Ca^{2+} -mediated signal transduction required for BDNF-induced collapse. By modulating the cAMP-dependent activity through the intrinsic programming or interaction with other factors present in the environment, a neuron thus could respond to the same extracellular factors with different morphological and cellular changes at different stages during development.

Key words: growth cone; collapse; neurotrophin; calcium; cAMP; guidance; repulsion

Formation of neuronal circuitry during development requires the growth and guidance of developing axons to their correct target cells. This process depends on the response of the growing tip of an axon, the growth cone, to a variety of extracellular factors present in developing embryos, including surface-bound as well as diffusible molecules (Bray and Hollenbeck, 1988; Keynes and Cook, 1995a; Tessier-Lavigne and Goodman, 1996). Guidance by positive cues that promote axonal extension and attract growth cones has been considered the main mechanism *in vivo*. However, recent studies have established an equally important role for inhibitory or repulsive cues in axonal guidance (Luo and Raper, 1994; Pini, 1994; Dodd and Schuchardt, 1995; Keynes and Cook, 1995b; Tessier-Lavigne and Goodman, 1996). By inhibiting axonal growth in "wrong" directions and by collapsing the growth cones entering inappropriate regions, these molecules can repel elongating axons and thus help them to reach the correct destination (Pini, 1993; Fan and Raper, 1995; Messersmith et al., 1995; Püschel et al., 1995).

Neurotrophins are a family of neurotrophic factors with profound influence on neuronal proliferation, survival, and differen-

tiation (for review, see Barde, 1990; Thoenen, 1991; Davies, 1994b; Klein, 1994; Snider, 1994; Lindsay, 1996). Recent studies have revealed a number of novel biological actions of neurotrophins on various aspects of developing nervous systems, including axonal (Diamond et al., 1992; Cohen-Cory and Fraser, 1995) and dendritic (Cohen-Cory et al., 1991; McAllister et al., 1995, 1996) morphology, synaptic activity (Lohof et al., 1993; Kim et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Figurov et al., 1996) and maturation (Wang et al., 1995), and synaptic patterning (Cabelli et al., 1995). A role for neurotrophins in the regulation of axonal growth is supported by the remarkable ability of neurotrophins to promote neurite outgrowth of sensitive populations of neurons (Snider and Johnson, 1989; Kuffler, 1994; Lundborg et al., 1994). Furthermore, the chemoattractive effect of nerve growth factor (NGF) was demonstrated previously *in vitro* (Lettourneau, 1978; Gundersen and Barrett, 1980) as well as *in vivo* (Menesini-Chen et al., 1978), suggesting a role for neurotrophins in axonal guidance. Despite subsequent evidence that NGF is not responsible for long-range growth cone guidance (Lumsden and Davies, 1983; Davies et al., 1987), a role for NGF in local regulation of axonal growth has been proposed (Gallo et al., 1997). Recent findings that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are expressed relatively early during development (Maisonpierre et al., 1990; Hallböök et al., 1993) further raise the possibility for neurotrophins to play a role in the early development of the nervous system. The demonstration of chemoattractive effects of BDNF and NT-3 in culture (Ming et al., 1997; Song et al., 1997) suggests that they could be candidates involved in axonal growth and guidance.

We now report a collapsing effect of neurotrophins on growth cones of embryonic *Xenopus* spinal neurons in early stages in

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culture. Acute application of BDNF induces rapid growth cone collapse followed by neurite retraction of *Xenopus* neurons in 6 hr cultures. The collapsing effect of BDNF depends on the activation of Trk receptor tyrosine kinase and requires an influx of extracellular Ca^{2+} . Furthermore, the Ca^{2+} -mediated BDNF-induced collapse is regulated by cytosolic cAMP-dependent activity; elevation or inhibition of cAMP-dependent activity blocked or enhanced the collapsing effect, respectively. Such cAMP-dependent regulation is found to be responsible for the disappearance of the collapsing effect of BDNF on neurons in 24 hr cultures. Our results suggest that neurotrophins may play important roles in the regulation of growth cone motility and extension; such regulation may well depend on the developmental stages of the neurons and could be influenced by other extracellular factors that modulate the cytosolic cAMP activity, leading to differential responses of the neuron to the same factor.

MATERIALS AND METHODS

Cell culture. Cultures of embryonic *Xenopus* spinal neurons were prepared according to procedures reported previously (Spitzer and Lamborghini, 1976; Tabti and Poo, 1990). The neural tube tissue from developing embryos at stage 20–22 (Nieuwkoop and Faber, 1967) was dissociated in a Ca^{2+} - and Mg^{2+} -free Ringer's solution supplemented with EDTA (in mM: 115 NaCl, 2.5 KCl, 10 HEPES, and 0.5 EDTA, pH 7.6), plated on clean glass coverslips, and incubated at room temperature (20–22°C). The culture medium consisted of 50% (vol/vol) Leibovitz medium (Life Technologies, Gaithersburg, MD), 1% (v/v) fetal bovine serum (Life Technologies), and 49% (v/v) Ringer's solution (in mM: 115 NaCl, 2 CaCl₂, 2.5 KCl, and 10 HEPES, pH 7.6).

Neurotrophins and chemicals. Human recombinant BDNF and NGF were generously provided by Regeneron Pharmaceuticals, (Tarrytown, NY). All neurotrophins were aliquoted at 10 mg/ml and stored at -85°C . Working stock solutions of neurotrophins at 100 $\mu\text{g}/\text{ml}$ were prepared and used within 1 week. Neurotrophins at working concentrations were prepared in culture medium before each experiment. A water-soluble derivative of forskolin (7-deacetyl-7-(*O*-*N*-methylpiperazino)- γ -butyryldihydrochloride), K252a, (R_p)-cAMP, (S_p)-cAMP, and staurosporine were all purchased from CalBiochem (La Jolla, CA). Cytochalasin D was purchased from Sigma (St. Louis, MO).

Collapsing experiments. Most of the experiments were performed on a Nikon TMS inverted microscope equipped with phase-contrast optics and a 20 \times objective (Nikon, Tokyo, Japan). The images of individual neurons were acquired through a $\frac{1}{3}$ inch CCD video camera (Coordinated Systems, East Hartford, CT) and digitized by a SNAPPY video digitizer (Play, Rancho Cordova, CA). For high-resolution imaging, cells grown on a glass coverslip were mounted on a microscopy chamber using silicon vacuum grease (Dow Corning, Midland, MI) and examined on a Nikon Diaphot 300 inverted microscope equipped with differential interference contrast (DIC) optics and a 40 \times , numerical aperture 1.3 oil-immersion objective. A $\frac{1}{2}$ inch CCD video camera (C2400-75i; Hamamatsu Photonics System, Bridgewater, NJ) was used for video imaging in conjunction with an Argus-20 image processor (Hamamatsu) for image enhancement. The video images were background-subtracted, averaged over four video frames, and contrast-enhanced in real time using Argus-20. The enhanced video images were digitized and acquired by a personal computer at a standard rate of one frame every 2 min, although faster rates were sometimes used to examine the dynamic changes of growth cone morphology. For all the experiments, a control period of 5 min observation was performed on each neuron to assess the normal neurite extension before the neurotrophin addition. Neurotrophins at their final working concentrations were applied to the culture by rapid perfusion of the culture medium. Immediately after the perfusion, 10 min of time-lapse recording at the standard rate was performed. For each pharmacological treatment, the cells were pretreated for 20 min with various chemicals before the addition of neurotrophins, and the effect of these chemicals alone on growth cone extension was monitored during the 20 min pretreatment. Only those chemicals that did not significantly affect growth cone extension were used for further experiments.

Image analysis was performed using the ImageTool program (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). The lengths of neurite processes were measured by tracing the entire trajectory of neurite extension, including all major branches. Because of the dynamic nature of filopodia and lamellipodia, they were not included in the length measurement. Therefore, the length measurement for each neurite process ends at the center of the phase-dark "palm" of the growth cone.

RESULTS

Neurotrophin collapsing effects

Isolated spinal neurons in 6 hr *Xenopus* cultures were used for these experiments. Active neurite extension at a rate of $\sim 15 \mu\text{m}/\text{hr}$ is observed in these cultures (Zheng et al., 1996b). Time-lapse video imaging was performed to monitor the changes in growth cone motility and neurite lengths before and after the addition of BDNF. In a typical experiment, a cell was monitored for 5 min before and 10 min after BDNF application. BDNF was applied to the culture by rapid perfusion with the culture medium containing the final concentration of BDNF. Exposure of cultured *Xenopus* neurons to 50 ng/ml BDNF caused rapid collapse of growth cones and subsequent withdrawal of neurite processes (Fig. 1). The collapsing response was observed as early as 1 min after BDNF addition, and the maximal neurite retraction was observed within the next 4 min. Whereas some neurons responded to BDNF by drastically withdrawing their processes (Fig. 1A), others displayed moderate neurite retraction and were able to resume extension after the washout of BDNF (Fig. 1B). Growth cones undergoing drastic collapse appeared to maintain some adhesion to the substratum, as evidenced by the thin membrane traces left behind (Fig. 1A, double arrowheads), suggesting that the collapse did not result from a complete loss of cell adhesion. The growth cone collapse induced by BDNF is accompanied by a number of morphological changes at the growth cone, most notably including the transient loss of filopodia (Fig. 1, arrows) and the later appearance of lamellipodial protrusion (arrowheads), an effect of BDNF that was also observed in 24 hr cultured *Xenopus* spinal neurons (Ming et al., 1997).

To quantify the collapsing effect, we directly measured the length of each neurite process at the beginning and at various times after the addition of BDNF. A growth cone is considered exhibiting collapsing response when it displays the morphological changes described above and withdraws at least 10 μm (approximately the average size of *Xenopus* growth cones) at any time point during the 10 min observation after exposure to BDNF. The percentage of growth cones exhibiting collapse was determined and used to assess the overall responsiveness of *Xenopus* growth cones to neurotrophins (Fig. 2A). To further quantitatively analyze the collapsing response of growth cones from a population of neurons, the length of each neurite process at various times after BDNF addition was measured and normalized against the length before BDNF application and presented as the percentage of original length (POL). POL did not exhibit a normal distribution; therefore, results from populations of neurons are presented as box whisker plots to illustrate the overall response from a population of cells (Fig. 2B). The boxes enclose the 25th and 75th percentiles, the horizontal lines mark the median, and the error bars denote the 10th and 90th percentiles of the distributions. The mean POL at various times after BDNF application is also presented on the same graph as symbol line plots. The majority of neurons responded to acutely applied BDNF by collapsing their growth cones followed by neurite withdrawal within 4 min after BDNF addition; the neurite retraction induced by BDNF was attenuated thereafter. Although some

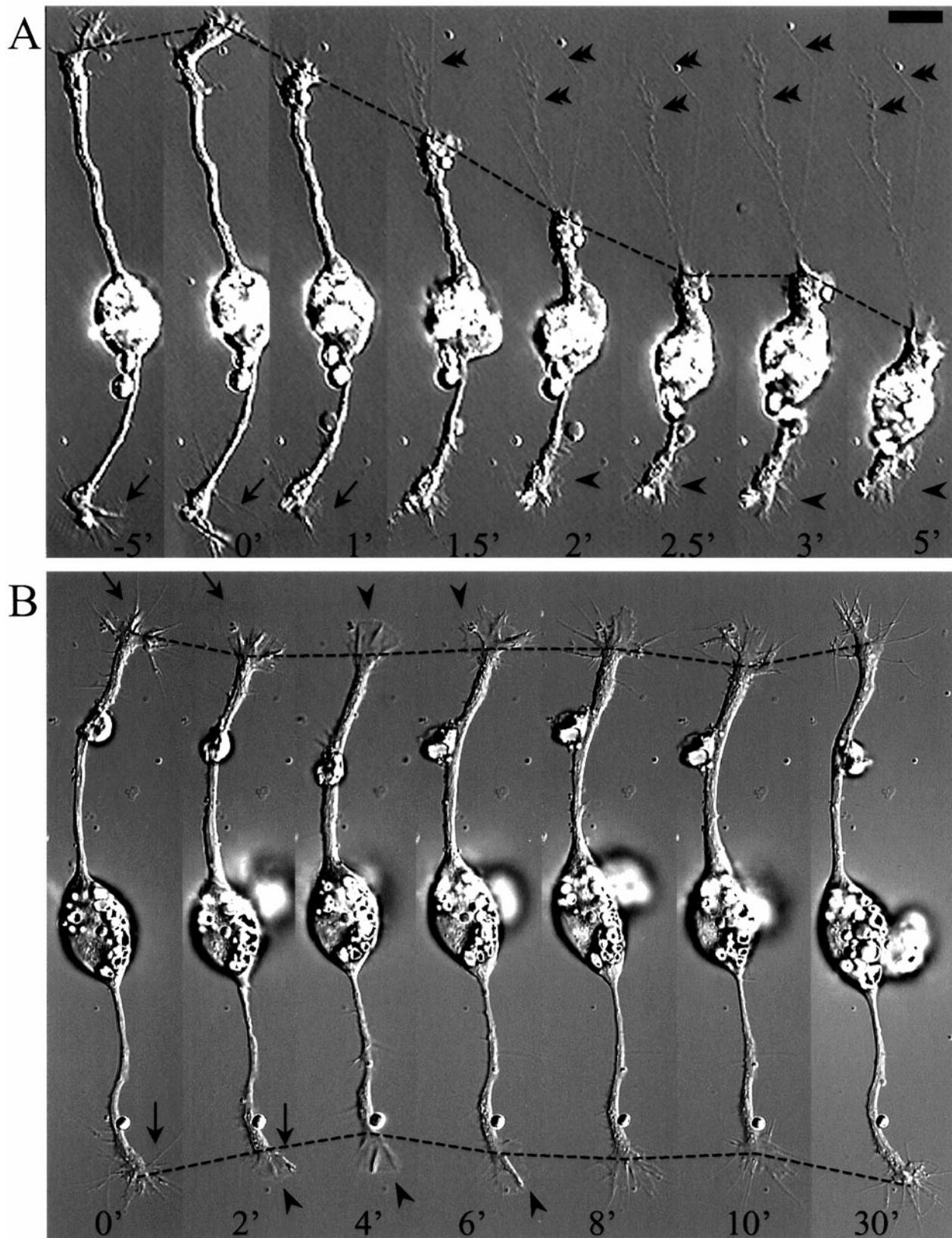


Figure 1. BDNF-induced collapse of growth cones of cultured embryonic *Xenopus* spinal neurons. Time-lapse DIC sequences showing growth cone collapse followed by dramatic (*A*) or moderate (*B*) neurite retraction induced by BDNF. Numbers represent minutes at various times before (*negative numbers*) and after (*positive numbers*) the application of 50 ng/ml BDNF (at time 0). In *B*, BDNF was washed out at 10 min after the application, and neurite extension was observed thereafter. Note the transient loss of filopodia (*arrows*) after BDNF application and the appearance of lamellipodial protrusion at later times (*arrowheads*). In *A*, also note that some adhesion remained during growth cone collapse (*double arrowheads*). Dashed lines depict the positions of the growth cones at various times. Scale bar, 15 μ m.

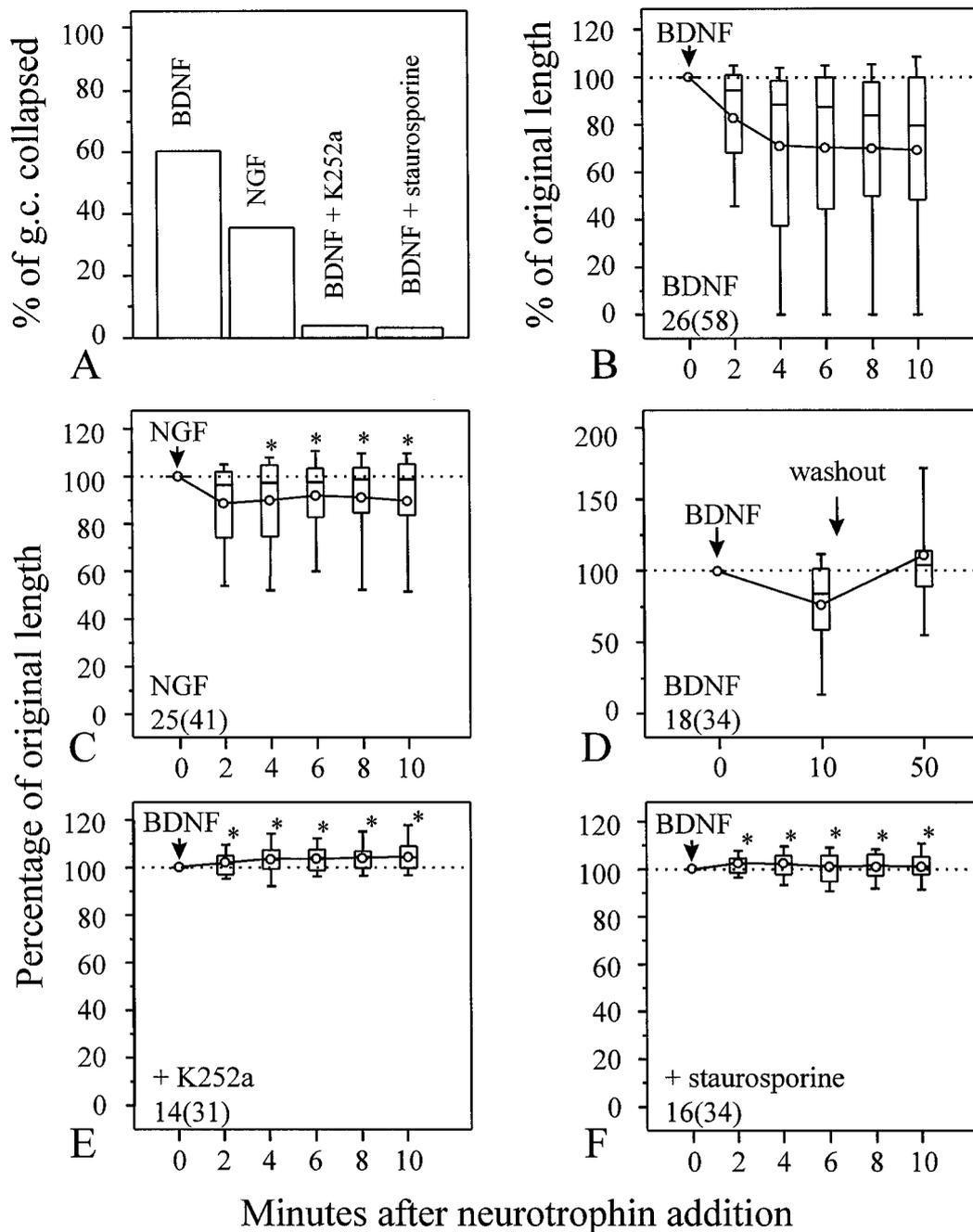


Figure 2. Quantitative analysis of growth cone collapse induced by neurotrophins. *A*, The percentage of growth cones collapsed in response to various neurotrophins is determined by measuring the lengths of each neurite at various times after the addition of neurotrophins. A growth cone is considered to exhibit collapse when it withdraws at least 10 μm , in addition to the morphological changes described in Results. *B–F*, The growth cone collapse was quantified by measuring the lengths of each neurite at various times after the addition of neurotrophins, normalized against the lengths before the neurotrophin addition, and presented as the percentage of original length (POL). POL did not follow a normal distribution; therefore, the data are presented as the box whisker plots. The boxes enclose the 25th and 75th percentiles; the horizontal lines mark the median; and the error bars denote the 10th and 90th percentiles of the distributions. Circles represent the mean POL. *B*, Acute application of 50 ng/ml BDNF induced rapid growth cone collapse and neurite retraction, but the related factor NGF had little effect on the neurite length (*C*). When compared with the POL of BDNF (*B*) at corresponding times after the neurotrophin addition, NGF caused significantly less collapse than BDNF ($*p < 0.05$, Mann–Whitney test). *D*, BDNF-induced growth cone collapse is reversible. When BDNF was washed out at the end of 10 min application, growth cones recovered and resumed extension. *E*, *F*, BDNF collapsing effect seems to be mediated by Trk receptor tyrosine kinases. Incubation of cells with 200 nM K252a (*E*) or 100 nM staurosporine (*F*) completely blocked BDNF-induced collapse [$*p < 0.0001$, Mann–Whitney test, compared with the POL of BDNF alone (*B*) at corresponding times after BDNF addition]. Numbers indicate the numbers of cells (growth cones) examined.

neurons largely withdrew their neurite processes within the 10 min period (indicated by the small POL at the 25th percentile: 41% at 4 min and 49% at 10 min after BDNF addition), most neurons only showed growth cone collapse with a small amount of neurite retraction (median POL, 88% at 4 min and 80% at 10 min), indicating selective responsiveness by different neurons. The collapsing effect seems to be specific for BDNF. The related factor, NGF, at the same concentration was ineffective in inducing collapse of the majority of growth cones (35% of growth cones showing collapse, compared with 60% for BDNF; see Fig. 2A). This is further evidenced by the near 100% median POL after NGF addition (Fig. 2C; median POL, 97% at 4 min and 99% at 10 min), which is significantly higher than that of BDNF alone ($*p < 0.05$ when compared at corresponding time points, Mann–Whitney test). The collapsing effect of BDNF appears to be reversible. Although regrowth of these retracted processes was not observed during the 10 min period, retracted neurite processes did reextend after BDNF washout (Fig. 2D).

Involvement of Trk receptor tyrosine kinase

Most of the biological effects of neurotrophins are believed to be mediated by the high-affinity Trk receptor tyrosine kinases: NGF activates TrkA, BDNF and NT-4 activate TrkB, and NT-3 activates TrkC (for review, see Chao, 1992; Barbacid, 1994; Dechant et al., 1994). Binding of neurotrophins to Trk receptors induces dimerization of the receptors followed by autophosphorylation, leading to cascades of signaling events (Kaplan and Stephens, 1994; Barbacid, 1995). The growth cone collapsing effect of BDNF appeared to be mediated by Trk receptors, because it was completely blocked by 200 nM K252a (Fig. 2A,E) or 100 nM staurosporine (Fig. 2A,F). These two potent, structurally related inhibitors for a broad range of serine and threonine protein kinases have been shown to effectively block the activity of Trk receptor tyrosine kinases (Knusel and Hefti, 1992; Nye et al., 1992; Tapley et al., 1992). All neurotrophins also bind to a low-affinity receptor p75, which could modulate Trk activity (Chao and Hempstead, 1995). The potential contribution of p75 receptors in the BDNF-induced growth cone collapse remains to be elucidated.

Ca²⁺ mediates the collapsing effect

Activation of Trk receptor tyrosine kinases by neurotrophins is known to elicit a range of second messenger responses, including increases in intracellular Ca²⁺ (Nikodijevic and Guroff, 1991; Berninger et al., 1993; De Bernardi et al., 1996; Stoop and Poo, 1996), cAMP (Knipper et al., 1993a,b), cGMP (Laasberg et al., 1988), and phosphoinositide turnover (Contreras and Guroff, 1987), as well as to activate the protein kinases Src, Raf, and the GTP-binding protein Ras (D'Arcangelo and Halegoua, 1993; Heumann, 1994; for review, see Kaplan and Stephens, 1994; Greene and Kaplan, 1995; Segal and Greenberg, 1996). Ca²⁺ and cAMP are two important second messengers that are known to affect growth cone motility and behavior (Kater and Mills, 1991; Kim and Wu, 1996). To test whether Ca²⁺ is involved in BDNF-induced growth cone collapse, we removed extracellular Ca²⁺ by using a Ca²⁺-free medium (in mM: 115 NaCl, 2.5 KCl, 2 MgCl₂, 1 EGTA, and 10 HEPES, pH 7.4). BDNF-induced growth cone collapse was completely blocked in the Ca²⁺-free medium (Fig. 3A–C). Results from a population of neurons show that growth cones actually extended in the Ca²⁺-free medium during the 10 min exposure to BDNF (Fig. 3C). The possibility that the removal of extracellular Ca²⁺ may have interfered with the activation of Trk receptors by BDNF is argued against by the finding that

BDNF-induced lamellipodial protrusion was still observed in the Ca²⁺-free medium (Fig. 3A,B; also see Ming et al., 1997), suggesting that the action of Ca²⁺-free medium on collapse is likely downstream of the receptor activation. To further test whether an influx of Ca²⁺ is involved in BDNF-induced growth cone collapse, we loaded the cells with BAPTA (10 μM BAPTA AM for 20 min), a rapid Ca²⁺ chelator (Tsien, 1980), before the application of BDNF. Loading neurons with BAPTA appeared to slow down the growth cone extension but did not cause growth cone collapse or retraction. However, the BDNF collapsing effect was diminished by BAPTA (Fig. 3D). Taken together, our results suggest that an influx of extracellular Ca²⁺ is required for the collapse, and that the Ca²⁺ signaling pathway mediates the collapsing effect of BDNF.

cAMP regulates the collapsing effect

We further tested the role of cAMP in BDNF-induced growth cone collapse. Application of 100 μM (*R_p*)-cAMP, a competitive inhibitor of protein kinase A (Rothermel and Parker Botelho, 1988; Dostmann et al., 1990), did not affect normal neurite extension, nor did it inhibit growth cone collapse induced by 50 ng/ml BDNF (Fig. 4B). Approximately 66% of (*R_p*)-cAMP-treated growth cones were collapsed by the addition of BDNF, a percentage not significantly different from that observed for growth cones exposed to BDNF alone (60%; see Fig. 2A). However, the collapsing effect of BDNF was completely blocked by 10 μM water-soluble derivative of forskolin (Fig. 4C, $*p < 0.05$, Mann–Whitney test), an agent known to stimulate membrane-bound adenylate cyclase, resulting in an elevation of [cAMP]_i (Laurenza et al., 1987). To further confirm that forskolin blockade of the collapse is directly associated with an elevation of [cAMP]_i, we applied (*S_p*)-cAMP, a membrane-permeant cAMP analog that is resistant to hydrolysis (Rothermel and Parker Botelho, 1988; Dostmann et al., 1990). Although not as effective as forskolin, application of 500 μM (*S_p*)-cAMP blocked BDNF-induced growth cone collapse (Fig. 4D, $*p < 0.05$, Mann–Whitney test). The blockade of the BDNF collapsing effect by the elevation of [cAMP]_i is further supported by the small percentage of growth cones collapsed after exposure to 50 ng/ml BDNF (14.5 and 19.4% for forskolin- and (*S_p*)-cAMP-treated growth cones, respectively, compared with 60% for growth cones exposed to BDNF only).

Although inhibition of protein kinase A did not significantly affect the collapse induced by 50 ng/ml BDNF, (*R_p*)-cAMP did exert influence on the collapse induced by BDNF at lower concentrations. For example, BDNF at 5 ng/ml was ineffective to induce growth cone collapse. The percentage of growth cones collapsed (25%) and the extent of neurite retraction induced by 5 ng/ml BDNF were small (Fig. 5A). When 100 μM (*R_p*)-cAMP was applied to the culture, the collapsing effect was potentiated (Fig. 5B). The percentage of growth cones collapsed in response to 5 ng/ml BDNF was also increased to 46% when growth cones were treated with (*R_p*)-cAMP. To better illustrate the potentiation of the BDNF collapsing effect by (*R_p*)-cAMP, the distribution of POL from populations of growth cones at 10 min after BDNF addition was plotted as a histogram. The presence of (*R_p*)-cAMP caused more growth cones to respond to BDNF by collapse, as indicated by an increased number of growth cones with smaller POL values when compared with that of 5 ng/ml BDNF alone (Fig. 5C, arrow). Statistical analysis further confirms the significance of the potentiation effect on BDNF-induced collapse by the inhibition of PKA (Fig. 5B, $*p < 0.05$, Mann–Whitney test).

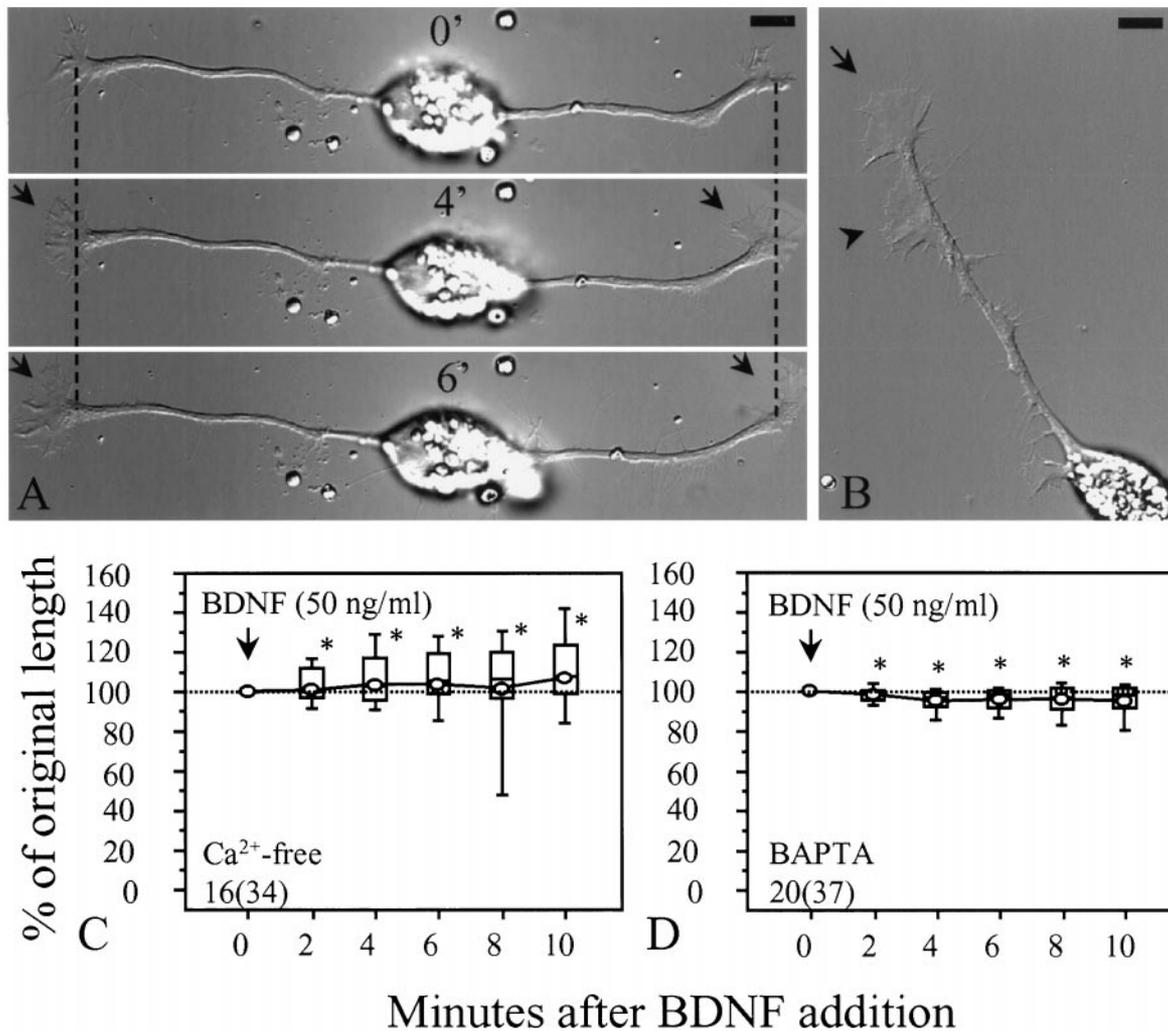


Figure 3. Ca^{2+} mediates the collapsing effect of BDNF. *A*, Representative images showing the changes induced by BDNF (50 ng/ml) in Ca^{2+} -free medium. No growth cone collapse and neurite retraction were observed after BDNF addition. However, BDNF-induced lamellipodial protrusion was still observed (arrows). Numbers represent minutes after BDNF addition. Dashed lines indicate corresponding positions along the neurite. *B*, BDNF-induced lamellipodial protrusion was observed not only at the growth cone (arrow) but also along the neurite shaft (arrowhead). The image was taken 10 min after BDNF application. *C*, Data from populations of cells in the Ca^{2+} -free medium are presented as a box whisker plot. Apparently no collapse was observed after BDNF application. *D*, Loading cells with 10 μM BAPTA AM for 20 min also blocked the collapsing effect of BDNF. No substantial changes in neurite length were observed. *Significantly different from the POL of BDNF in culture medium (Fig. 2*B*) at corresponding times after BDNF addition ($p < 0.001$, Mann–Whitney test). Numbers in *C* and *D* indicate the numbers of cells (growth cones) examined. Scale bars, 10 μm .

Loss of collapsing response of old neurons to BDNF in 24 hr cultures

The collapsing effect of BDNF was only observed on neurons in 6–8 hr cultures in which motile growth cones and active neurite extension are observed. In 24 hr cultures, although active neurite extension was still observed but at a slightly reduced rate (Ming et al., 1997), no apparent collapsing effect of BDNF was observed (Fig. 6*A*). Conversely, BDNF was found to elicit extensive lamellipodial protrusion along the neurite shaft and to induce chemoattractive turning of the growth cone (Ming et al., 1997; Song et al., 1997). These results suggest that neurons may respond to the same tropic factors differently at different developmental stages. Because a clear cAMP regulation of BDNF-induced collapse was observed in 6 hr cultures, it is possible that neurons in 24 hr cultures may have higher resting levels of endogenous cAMP-dependent activity, which could block the collapsing effect of BDNF. To test this hypothesis, we applied (R_p)-cAMP (100 μM) to 24 hr cultures to inhibit the activity of PKA. When 50 ng/ml

BDNF was applied to these (R_p)-cAMP-treated neurons, significant collapse of growth cones was observed in these 24 hr cultures (Fig. 6*B*, $*p < 0.001$, Mann–Whitney test). It appears that (R_p)-cAMP restored the collapsing response of 24 hr cultures to a level similar to that of 6 hr cultures. The percentage of growth cones collapsed by 50 ng/ml BDNF was also increased from 25 to 81% after growth cones were treated with (R_p)-cAMP in 24 hr cultures. These results suggest that the loss of the collapsing response to BDNF by neurons in 24 hr cultures is tightly associated with the endogenous cAMP-dependent activity, which may be elevated during the development.

Involvement of the actin cytoskeleton in BDNF-induced growth cone collapse

Although the signaling events downstream of second messengers are unknown, the actin cytoskeleton seems to be one of the final targets in BDNF-induced growth cone collapse. Cytochalasins are fungal metabolites that bind to the barbed ends of actin

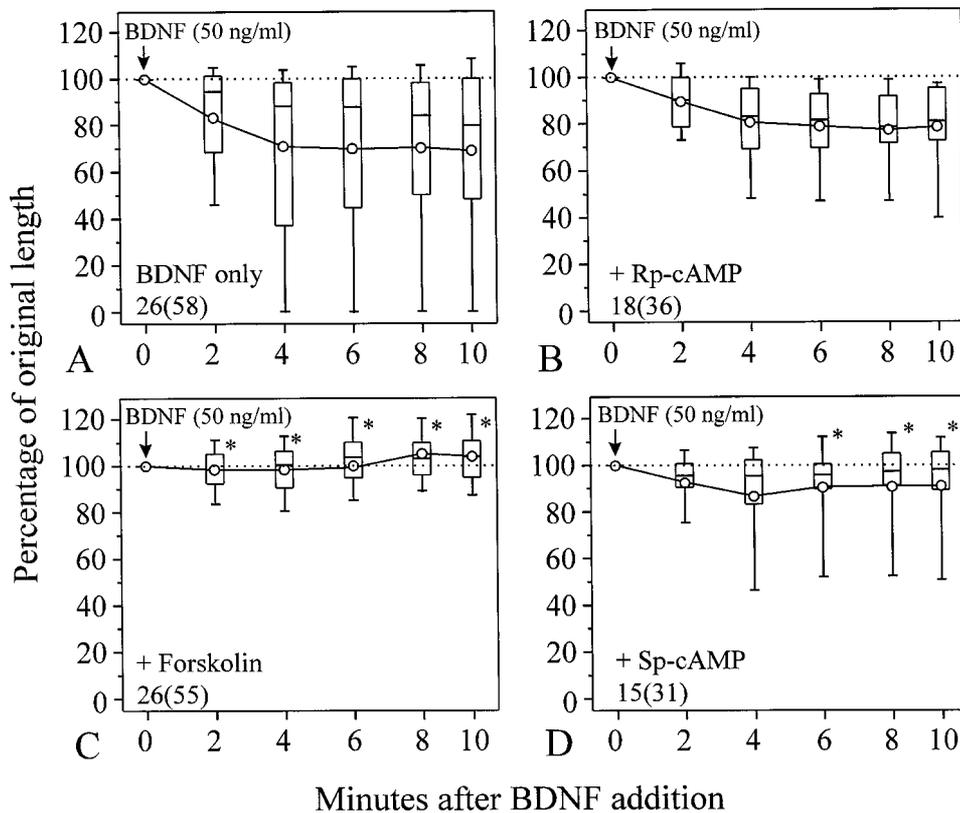


Figure 4. cAMP regulation of BDNF-induced growth cone collapse. *A*, Growth cone collapse induced by BDNF alone (from Fig. 2*B*). *B*, The culture was preincubated with 100 μ M (R_p)-cAMP for 20 min before BDNF application, and (R_p)-cAMP was present throughout the experiment. No significant difference from *A* was detected ($*p > 0.5$, Mann-Whitney test). *C*, *D*, Application of 10 μ M forskolin (*C*) or 500 μ M (S_p)-cAMP (*D*) blocked the collapsing effect. Both chemicals were preincubated with the culture for 20 min before the application of BDNF and present throughout the experiment. Numbers represent numbers of cells (growth cones) examined. *Significantly different from BDNF alone (*A*) at corresponding times ($p < 0.05$, Mann-Whitney tests).

filaments to prevent further polymerization, leading to the disassembly of actin filaments over time. Application of cytochalasin D at 1 μ M did not cause neurite retraction but effectively abolished BDNF-induced growth cone collapse (Fig. 7) as well as lamellipodial protrusion (Ming et al., 1997). Dependence on actin polymerization has been observed previously in NGF-induced rapid neurite retraction of cultured sensory neurons (Griffin and Letourneau, 1980). It was suggested that extensive membrane ruffling induced at such thin neurite processes by growth factors may directly cause neurite retraction (Griffin and Letourneau, 1980), mainly as the result of cytoskeletal reorganization. However, the presence of lamellipodial protrusion and the blockade of collapsing effect in the Ca^{2+} -free medium (Fig. 3) argue strongly against this possibility. Other actin-based mechanism(s) must be involved in the collapse.

DISCUSSION

Acute, inhibitory effects of neurotrophins on growth cone motility

An increasing number of studies have established a “positive” role for neurotrophins in axonal and dendritic growth. In addition to the key activity of neurotrophins in promoting neurite outgrowth of responsive neuronal populations, neurotrophins have been shown to increase axonal sprouting (Diamond et al., 1992; Cohen-Cory and Fraser, 1995) and to regulate morphological development of dendrites (Cohen-Cory et al., 1991; McAllister et al., 1995, 1996). In our *Xenopus* cultures, addition of neurotrophins enhances neuronal survival and promotes neurite outgrowth of embryonic spinal neurons; when applied as concentration gradients, BDNF and NT-3 also exert chemotropic effects on growth cones in 18–24 hr cultures (Ming et al., 1997; Song et al., 1997). In this study, we provide evidence on a “negative” effect of

BDNF on growth cone extension of embryonic *Xenopus* neurons in 6 hr cultures: acute application of BDNF induces rapid growth cone collapse and neurite retraction. The effective neurotrophin concentrations for growth cone collapse are similar to those known to promote neuronal survival as well as to elicit a number of acute effects on these neurons (Ming et al., 1997). The collapsing effect appears to be relatively specific for BDNF, because NGF was ineffective in inducing growth cone collapse. Only a small portion of growth cones collapsed in response to NGF (35% of total growth cones examined, compared with 60% for BDNF). The lack of the collapsing effect of NGF is further demonstrated by the significantly smaller extent of neurite retraction than that of BDNF (Fig. 2*C*). Given the heterogeneity of *Xenopus* cultures, it is not surprising for the existence of subpopulations of neurons that may respond to different neurotrophins differently (Lohof et al., 1993; Ming et al., 1997). Nevertheless, the significant difference between BDNF and NGF in the ability of inducing growth cone collapse further supports the conclusion that the high-affinity Trk receptor (TrkB for BDNF), rather than the low-affinity p75 receptor, mediates the collapsing effect; otherwise, similar extents of growth cone collapse would have been observed for BDNF and NGF, because both can activate p75 effectively. However, our results do not exclude the possibility that p75 may be involved, but not required, in the collapsing action of neurotrophins. The possible contribution from the p75 receptor activation in BDNF-induced growth cone collapse remains to be determined.

Ca^{2+} is an important second messenger involved in regulation of a wide range of cellular activities, including growth cone motility (Kater and Mills, 1991; Zheng et al., 1996a). In BDNF-induced growth cone collapse, an influx of Ca^{2+} is required for the collapse, suggesting that the Ca^{2+} -dependent signaling path-

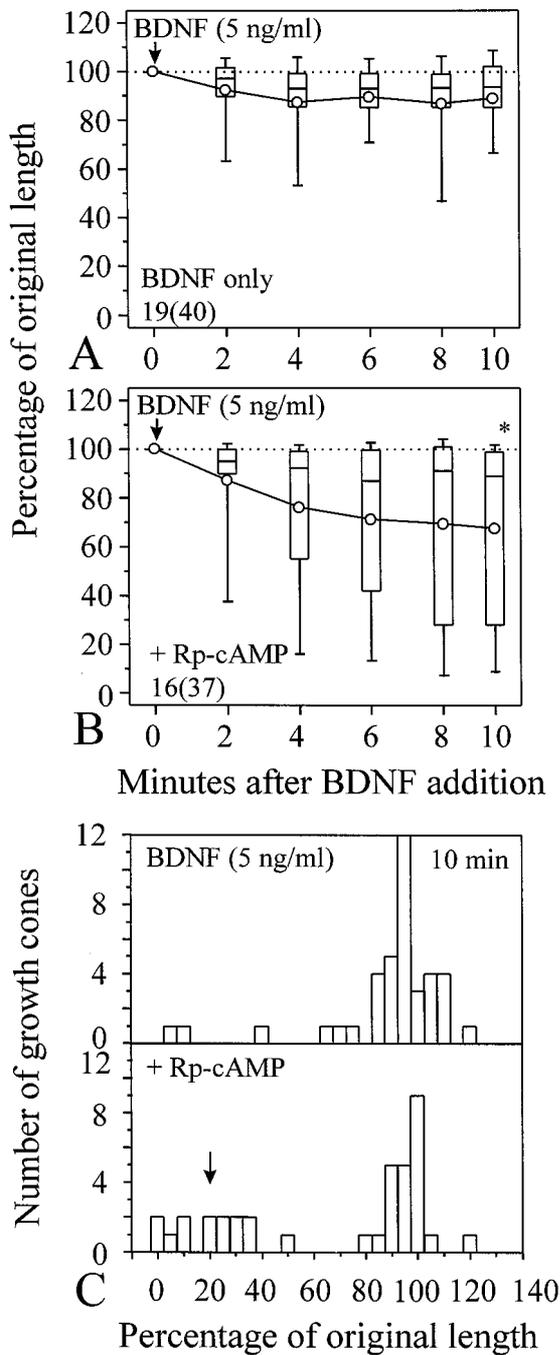


Figure 5. Potentiation of BDNF-induced growth cone collapse by (R_p) -cAMP. *A*, Growth cone collapse induced by 5 ng/ml BDNF alone. *B*, One hundred micromolar (R_p) -cAMP was added to the culture 20 min before the application of 5 ng/ml BDNF. Increased collapse was observed [$*p < 0.05$, Mann-Whitney test, compared with the POL of BDNF alone (*A*) at the corresponding time]. Numbers in *A* and *B* represent numbers of cells (growth cones) examined. *C*, Histogram showing the difference in the distribution of POL between BDNF alone (*top panel*) and BDNF with (R_p) -cAMP treatment (*bottom panel*) at 10 min after BDNF application (5 ng/ml). Presence of (R_p) -cAMP in the medium caused more growth cones to respond to BDNF with collapse, as evidenced by the appearance of a peak of the POL distribution at smaller POL (*arrow*).

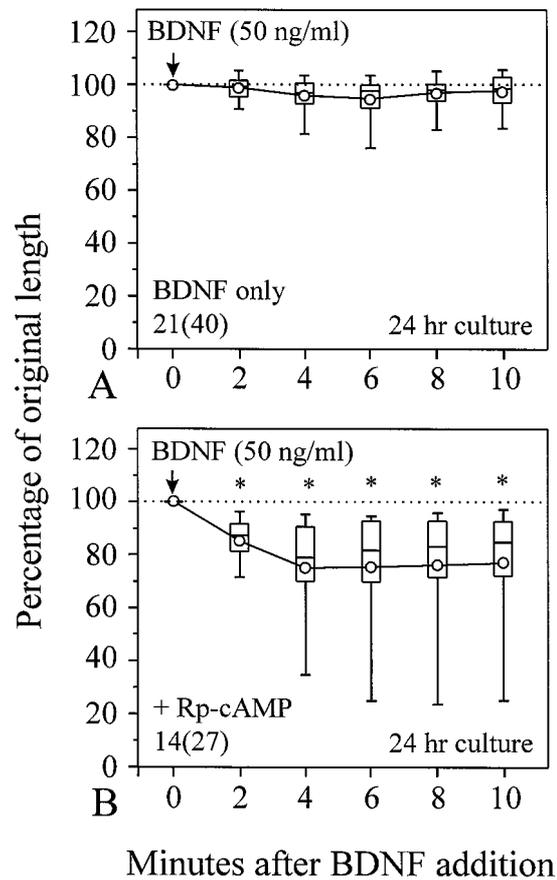


Figure 6. Endogenous cAMP-dependent activity plays a key role in the disappearance of the BDNF collapsing effect on neurons in 24 hr cultures. *A*, BDNF was apparently ineffective in inducing growth cone collapse in 24 hr cultures. However, when endogenous cAMP-dependent activity was inhibited by 100 μ M (R_p) -cAMP, the collapsing effect of BDNF was clearly observed (*B*). *Significantly different from the data in *A* at corresponding times ($p < 0.001$, Mann-Whitney test).

way mediates the collapse. Furthermore, the collapsing response is regulated by cAMP-dependent activity, because elevation or inhibition of cytosolic cAMP-dependent activity attenuated or potentiated the collapse, respectively. This cAMP-mediated regulation appears to play a key role in the loss of the collapsing response to BDNF by old neurons in 24 hr cultures. The endogenous cAMP-dependent activity in these neurons may be elevated to a higher level than that in neurons from 6 hr cultures, therefore leading to the blockade of the collapsing effect of BDNF. Our finding that inhibition of PKA by (R_p) -cAMP restored the collapsing response of these old neurons confirms such a notion. The cellular mechanism underlying the elevated levels of cAMP-dependent activity in these old neurons remains to be studied. Nevertheless, these results thus resolve the apparent contradiction between the negative collapsing effect and the positive effects of neurotrophins on neurite growth observed on same *Xenopus* cultures. By modulating its cytosolic levels of cAMP-dependent activity at different stages during development, a neuron could thus exhibit different responses to the same extracellular signals.

Although the cellular mechanism underlying BDNF-induced growth cone collapse is largely unknown at this moment, the actin cytoskeleton seems to play an important role. The result that cytochalasin D completely blocked the collapse induced by

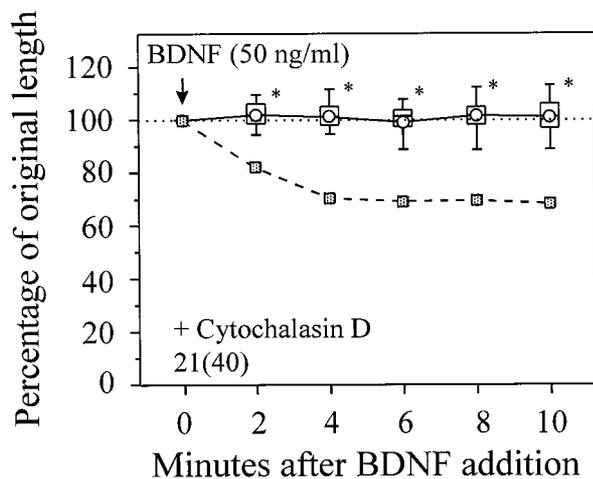


Figure 7. Cytochalasin D blocks growth cone collapse induced by BDNF. The mean POL from growth cones exposed to BDNF alone (Fig. 2B) is presented as the reference (squares, dashed line). Cytochalasin D at 1 μ M did not reduce the neurite length but blocked the collapsing effect of BDNF. *Significantly different from that of BDNF alone (Fig. 2B) at corresponding times ($p < 0.05$, Mann–Whitney test).

BDNF suggests that actin polymerization may be required for the collapse. However, in growth cone collapse induced by collapsin-1, actin depolymerization was observed (Fan et al., 1993), indicating that the collapse induced by BDNF and collapsin-1 may involve different cellular processes. On the other hand, cytochalasins are known not only to prevent actin polymerization but also to disrupt the existing actin cytoskeletal network. Therefore, the blockade of BDNF-induced collapse by cytochalasins could also suggest that an intact actin network is required for BDNF collapsing action, either for signal transduction or for force generating. In neurite retraction induced by lysophosphatidate (LPA) and thrombin, a similar dependence on the actin cytoskeleton was observed, and an actomyosin contractile mechanism was suggested to mediate the neurite retraction (Jalink and Moolenaar, 1992; Jalink et al., 1993). However, whether the same mechanism operates in BDNF-induced growth cone collapse and neurite retraction remains to be determined.

Mechanism(s) underlying the cAMP regulation of BDNF-induced collapse: a tentative model

The cAMP pathway has been suggested to regulate the activity of other signal transduction pathways (for review, see Iyengar, 1996). In smooth muscle, contractility is regulated by myosin light-chain kinase (MLCK), which can be phosphorylated by PKA, cGMP-dependent protein kinases, and Ca^{2+} -calmodulin-dependent protein kinase II (for review, see Burrige and Chrzanowska-Wodnicka, 1996). Phosphorylation of MLCK decreases its affinity for Ca^{2+} and calmodulin, inhibiting MLCK activity and contractility (Nishikawa et al., 1984; Tansey et al., 1994). It was suggested that LPA-induced neurite retraction is mediated by the contraction of cortical actin cytoskeleton, which may also be subjected to this kind of regulation (Tigyi et al., 1996). We hypothesize a similar cAMP-dependent regulation in BDNF-induced growth cone collapse (see Fig. 8). Elevation of [cAMP], in these cultured *Xenopus* neurons activates protein kinase A, which in turn phosphorylates the unknown protein “X” that might be involved in the main signaling pathway (Ca^{2+} -mediated) in BDNF-induced collapse. The phosphorylation inactivates X and blocks the collapse; dephosphorylation of X by phosphatases could reactivate the

protein. The resting levels of PKA and phosphatase activity in the cell are likely to reach a balance, leading to an equilibrium between active and inactive forms of X. Inhibition of PKA thus can change the equilibrium and increase the level of active X, leading to the potentiation of the collapsing effect induced by low concentrations of BDNF, which was observed in this study. At high concentrations of BDNF, the collapsing effect may already be saturated, and further enhancement by PKA inhibitors cannot be produced. Although our hypothesized model places cAMP-mediated regulation downstream of Ca^{2+} , it is possible that such cAMP regulation is upstream of Ca^{2+} ; that is, X mediates BDNF-induced Ca^{2+} influx to induce collapse that can be regulated by cAMP-dependent activity in a similar matter.

Collapsing effect and growth cone guidance: the *in vivo* relevance

Recent studies have identified an increasing number of receptor tyrosine kinases involved in axonal guidance (Tessier-Lavigne and Goodman, 1996). Although no *in vivo* evidence is available for a role of neurotrophic factors in axonal guidance, the chemoattractive effects of neurotrophins in culture suggest such a potential (Letourneau, 1978; Menesini-Chen et al., 1978; Gundersen and Barrett, 1980; Ming et al., 1997; Song et al., 1997). The collapsing effect observed here, together with previous observations of similar inhibitory effects of neurotrophins *in vivo* and in cell culture (Griffin and Letourneau, 1980; Zhang et al., 1994; Paves and Saarma, 1997), suggests that neurotrophins may exert acute, negative regulation on neuronal development. The observation that BDNF administered through a micropipette could also produce a similar inhibitory effect on *Xenopus* growth cones in 6 hr cultures (Q. Wang and J. Q. Zheng, unpublished observation) suggests that the negative regulation of axonal growth could be achieved by neurotrophins derived from point sources, such as those neurotrophin-expressing cells along developing axonal pathways, as well as target tissues (Elkabes et al., 1994; Hallböök et al., 1995). Such negative regulation of axonal growth, if occurring in the early stages during neuronal development *in vivo*, could be used to temporarily halt axonal extension to prevent developing axons from entering incorrect intermediate regions and targeting prematurely; by switching the dependence on specific neurotrophins (Davies, 1994a) or by modulating its endogenous cAMP activity in the later stages of development, a growth cone could then change its responsiveness to accommodate the positive influence of neurotrophins (O'Connor et al., 1990; Kuhn et al., 1995; Gallo et al., 1997).

The cAMP-dependent activity has recently been shown to regulate the turning direction of *Xenopus* growth cones exposed to BDNF gradients in 18–24 hr cultures (Song et al., 1997). A gradient of BDNF normally induces a chemoattractive response in these neurons (Ming et al., 1997; Song et al., 1997). It was proposed that a cytoplasmic Ca^{2+} gradient induced by the BDNF gradient produces the repulsive turning of the growth cone, which is normally overridden by a Ca^{2+} -induced cAMP gradient that attracts growth cones. Inhibition of cAMP-dependent activity thus results in an opposite turning response. Because the repulsive turning of growth cones is thought to result from a local, asymmetric collapse of the growth cone (Fan and Raper, 1995), our finding of the collapsing effect of BDNF provides the direct evidence to support the repulsive action of BDNF on embryonic *Xenopus* neurons. However, our data show that such a collapsing and repulsive effect of BDNF is mainly blocked by the change of endogenous cAMP-dependent activity in these

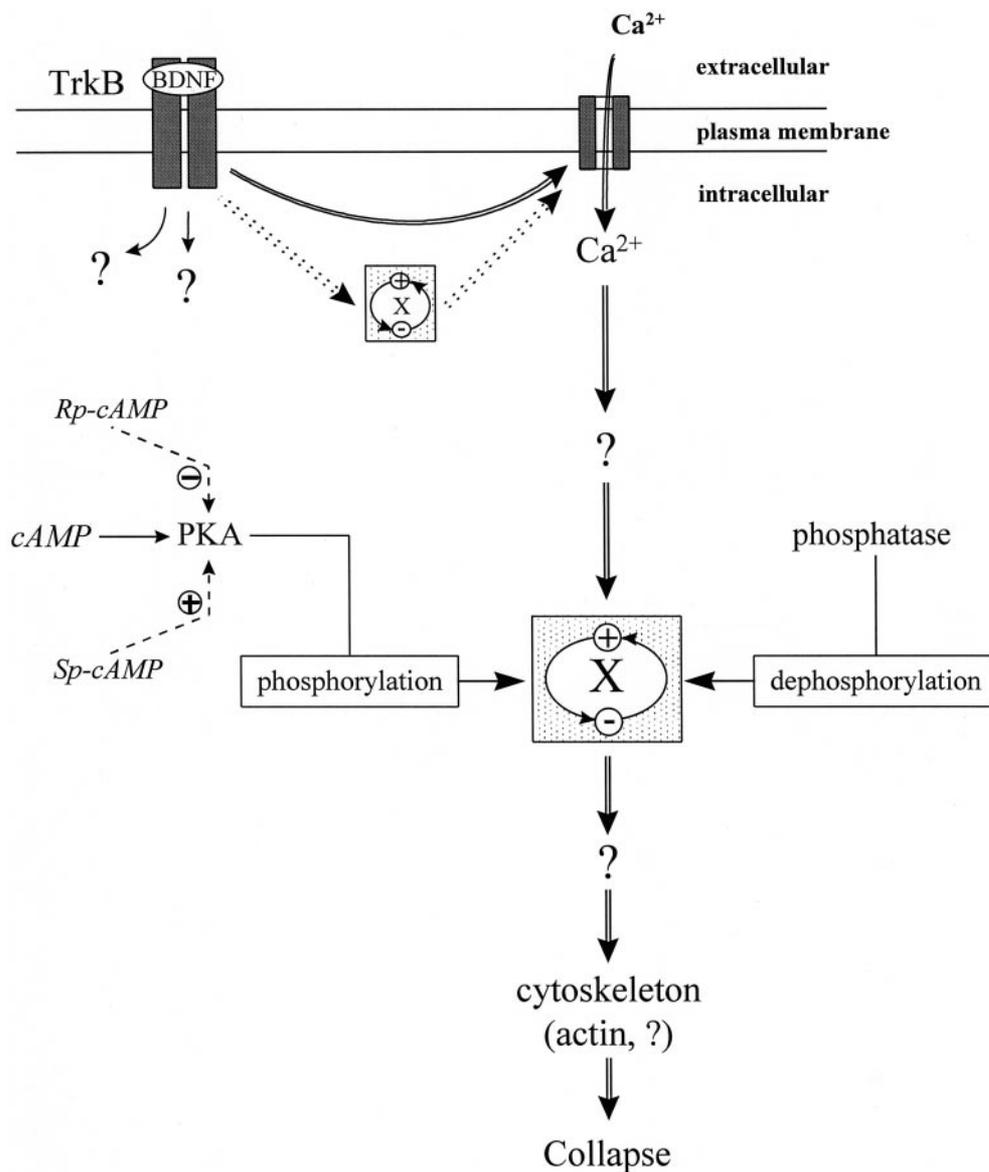


Figure 8. Schematic diagram showing the proposed model involved in the cAMP regulation of BDNF-induced collapse. Activation of TrkB receptors by BDNF induces multiple intracellular signaling cascades including a Ca²⁺ influx through Ca²⁺ channels, and the Ca²⁺ pathway mediates the collapsing effect (double lines with arrows). Although the unknown protein X is involved in the main Ca²⁺-signaling pathway for collapse, its activity can be modulated by phosphorylation and dephosphorylation by cAMP-dependent activity (single lines with arrows). PKA phosphorylates and inactivates (–) X, whereas phosphatase(s) dephosphorylate(s) and activate(s) (+) X. An equilibrium between the active and inactive X is likely reached in the cell. Experimental manipulation of PKA activity by (R_p)-cAMP or (S_p)-cAMP (dashed lines with arrows) can change the equilibrium and thus regulate the collapsing effect of BDNF. Activation of PKA by (S_p)-cAMP causes more X to become inactive, leading to the blockade of the collapsing effect; inhibition of the resting level of PKA activity by (R_p)-cAMP results in more active X, leading to the potentiation of the collapsing effect induced by the low concentrations of BDNF. Alternatively, the cAMP-mediated regulation can be upstream of Ca²⁺. In this case, the protein X mediates BDNF-induced Ca²⁺ influx required for growth cone collapse (dotted double lines with arrows), and its activity can be regulated by cAMP-dependent activity in a similar way.

18–24 hr neurons; the Ca²⁺-induced cAMP gradient, on the other hand, might be responsible for inducing attractive turning of the growth cone in BDNF gradients after the repulsive effect is blocked. When endogenous cAMP-dependent activity is inhibited in these 24 hr cultures, the collapsing (this study) and chemorepulsive (Song et al., 1997) effect of BDNF is observed. The cAMP-mediated regulation observed in this study might also play a role in repulsive guidance of growth cones by other molecules. Our preliminary study has shown that elevation of [cAMP]_i by forskolin completely blocked the repulsive turning of *Xenopus* growth cones induced by extracellular gradients of semaphorin III in culture (Q. Wang and J. Q. Zheng, unpublished observations), suggesting that the cAMP-dependent activity could regulate the repulsive action of semaphorins. Developing neurons *in vivo* do encounter many extracellular factors that act through second messenger-dependent mechanisms, including the cAMP-dependent pathway. Concurrent presence or absence of these factors may thus regulate the different responses of growth cones to the guidance cues. Furthermore, a neuron may switch on or off the pathways responsible for different cellular responses to the

same guidance cues by its intrinsic programming, allowing different responses at different stages during development.

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