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Electrical Activity Modulates Growth Cone Guidance by Diffusible Factors

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Summary

Brief periods of electrical stimulation of cultured *Xenopus* spinal neurons resulted in a marked alteration in the turning responses of the growth cone induced by gradients of attractive or repulsive guidance cues. Netrin-1-induced attraction was enhanced, and the repulsion induced by myelin-associated glycoprotein (MAG) or myelin membrane fragments was converted to attraction. The effect required the presence of extracellular Ca^{2+} during electrical stimulation and appeared to be mediated by an elevation of both cytoplasmic Ca^{2+} and cAMP. Thus, electrical activity may influence the axonal path finding of developing neurons, and intermittent electrical stimulation may be effective in promoting nerve regeneration after injury.

Introduction

Path finding of growing axons and the formation of initial connections in the developing nervous system are generally thought to be processes independent of electrical activity (Shatz and Stryker, 1988; Goodman and Shatz, 1993). However, recent findings have shown that electrical activity is required for growing thalamic axons to reach their appropriate cortical target area (Catalano and Shatz, 1998) and for axons of cortical pyramidal neurons to form layer-specific connections (Dantzker and Callaway, 1998). Furthermore, some peripheral olfactory projections are affected in mice deficient in a cyclic nucleotide-gated channel subunit, suggesting that path finding of these axons is in part influenced by odorant-dependent activity (Zheng et al., 2000). In order to test directly the effect of electrical activity on the guidance behavior of the nerve growth cone in a controlled environment, we examined the response of the growth cone of cultured *Xenopus* spinal neurons to microscopic gradients of diffusible guidance molecules immediately after electrical stimulation of the neuron. We found that a gradient of netrin-1 normally induces repulsive turning of growth cones of spinal neurons in young cultures (<10 hr in culture) and attractive turning of these growth cones in older cultures (>16 hr in culture). Pre-exposure of the culture to brief electrical stimulation resulted in the conversion of repulsion to attraction in young cultures and enhanced the attraction in older cultures in response to gradients of netrin-1. Moreover, repulsion of growth cones induced by myelin-associated glycoprotein (MAG) or myelin membrane fragments in older cultures was converted to attraction. The effects of electrical stimulation required the presence of extracellular Ca^{2+} during stimulation and appeared to be mediated by an elevation of cytoplasmic cAMP. These results provide a direct demonstration that brief electrical activity in the neuron can markedly alter the guidance behavior of nerve growth cones.

Results

Modulation of Netrin-1-Induced Responses

We first examined the response of cultured *Xenopus* spinal neurons (Tabti et al., 1998) to a gradient of netrin-1, a guidance cue known to serve as either an attractant or a repellent in different systems (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Song and Poo, 1999). The gradient was established across the growth cone of an isolated neuron by repetitive pulsatile ejection of picoliters of solutions containing netrin-1 from a micropipette (Lohof et al., 1992; Song et al., 1997, 1998). The tip of the micropipette was positioned at a distance of 100 μm from the growth cone and at an angle of 45° with respect to the original direction of neurite extension. Previous work has shown that for spinal neurons in 14–20 hr cultures, growth cones exhibit attractive turning responses toward the source of netrin-1 when the concentration of netrin-1 in the micropipette is above 2.5 μg/ml (Ming et al., 1997b) (see Figure 1A). When the netrin-1 concentration was lowered to 1 μg/ml (corresponding to about 1 ng/ml at the growth cone) (Ming et al., 1997b), no turning response was observed (Figure 1B). However, when a 16–20 hr culture was first exposed to pulsatile electrical stimuli (duration 2 ms, 10 pulses at 2 Hz; see Experimental Procedures), which were capable of triggering action potentials in the neuron, the growth cone exhibited marked attractive turning in the same gradient produced by the low dose of netrin-1 (Figure 1C). Thus, electrical stimulation had enhanced the sensitivity of the growth cone to netrin-1.

The response of these *Xenopus* growth cones to a netrin-1 gradient undergoes marked developmental changes, a phenomenon similar to that previously described for the effects of brain-derived neurotrophic factor (BDNF) (Wang and Zheng, 1998). As shown in Figure 1D, the netrin-1 gradient (5 μg/ml in the pipette) induced repulsive turning responses of growth cones of young spinal neurons at 6–10 hr after plating, although the

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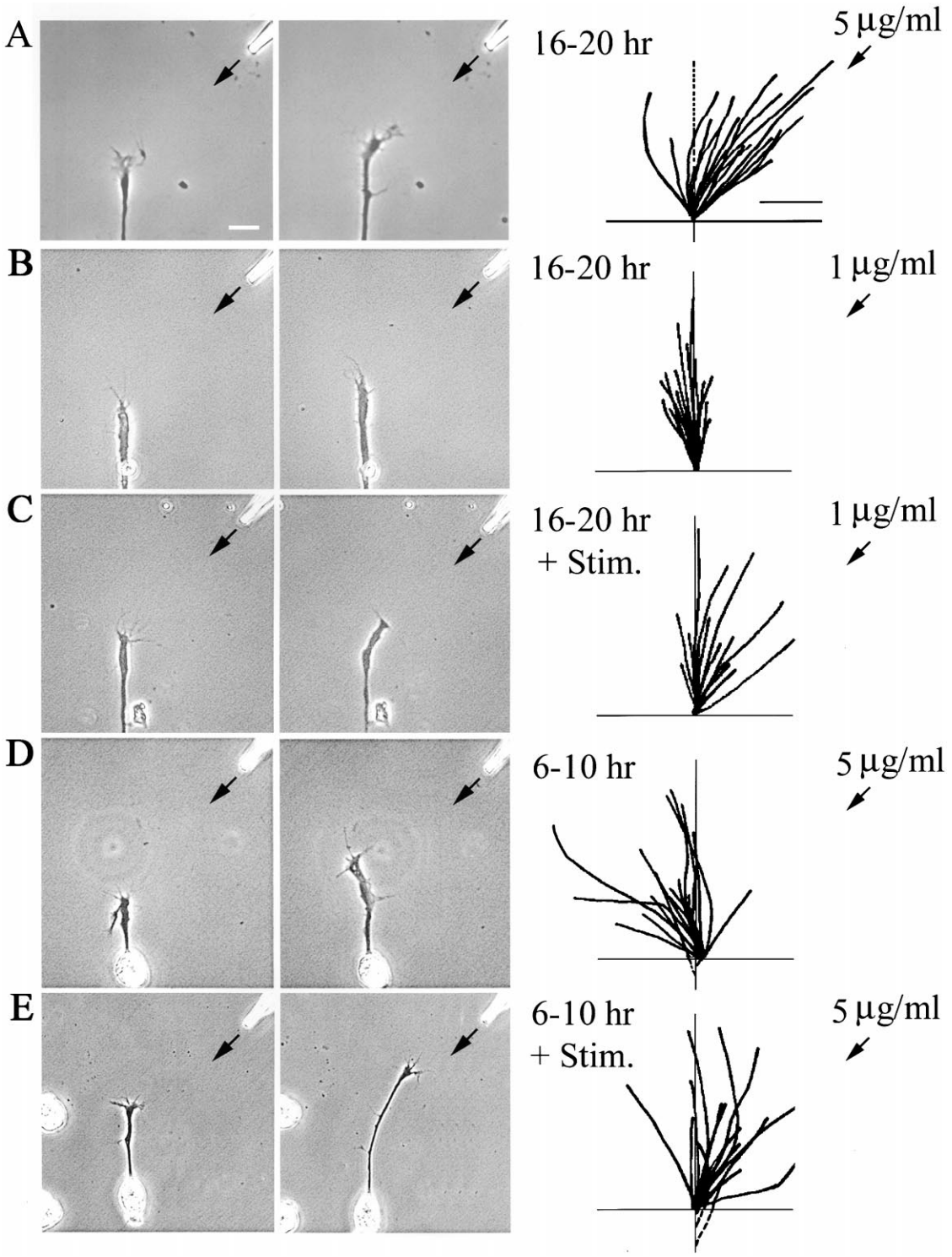

Figure 1. Modulatory Effects of Electrical Stimulation on Growth Cone Turning Induced by Gradients of Netrin-1

(A) A gradient of netrin-1 was applied to the growth cone by pulsatile application of a solution containing netrin-1 (5 μg/ml) from a micropipette. Micrographs were recorded at the onset (left) and the end (right) of a 1 hr exposure to a netrin-1 gradient for a neuron in a 16–20 hr culture. Bar = 20 μm. Superimposed traces on the right depict the trajectory of neurite extension during the 1 hr period for all the neurons examined under these conditions. The origin is the center of the growth cone at the onset of the experiment, and the original direction of growth was vertical. The arrow indicates the direction of the gradient. Bar = 10 μm.

(B) Experiments carried out in the same manner as in (A), except that the concentration of netrin-1 in the pipette was reduced to 1 μg/ml.

(C) Experiments carried out as in (B), except that the culture was pre-exposed to a brief period of field stimulation (10 pulses at 2 Hz).

(D and E) Experiments similar to that shown in (B) and (C), respectively, except that young neurons (6–10 hr cultures) were subjected to a gradient of a higher dose of netrin-1 (5 μg/ml in the pipette). The dashed traces depict cases in which the neurite shank shifted during the 1 hr assay.

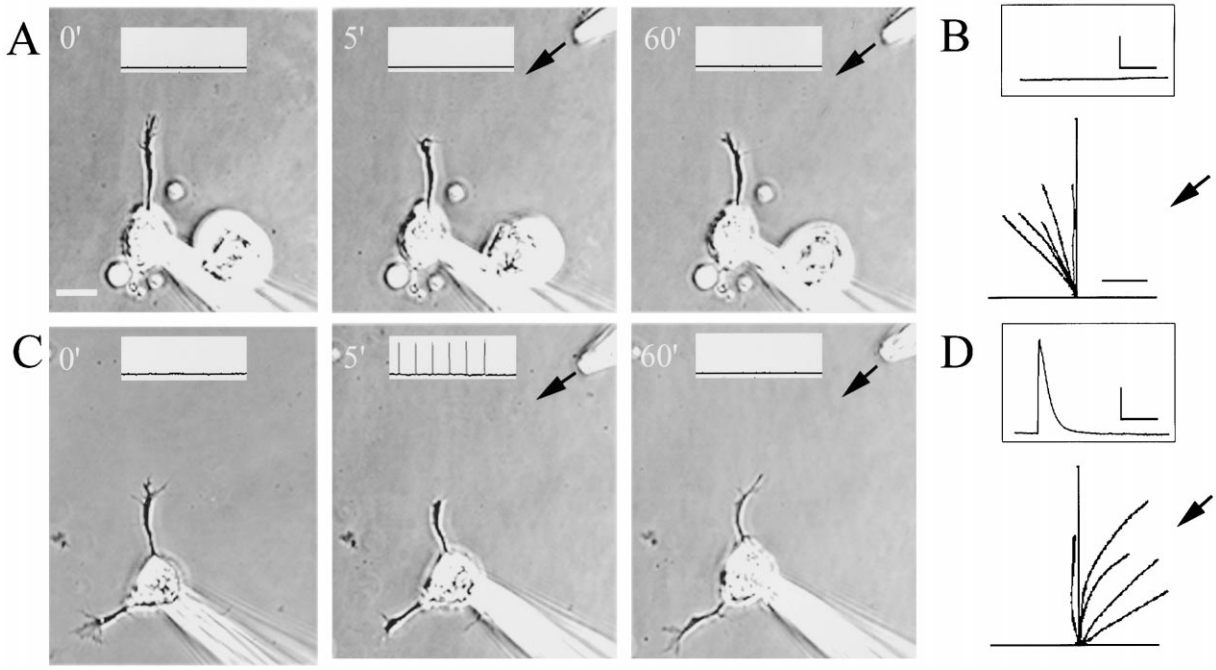

Figure 2. Modulation of the Turning Responses of Growth Cones to Netrin-1 by Intracellular Electrical Stimulation

Perforated whole-cell recordings were made from the soma of spinal neurons in 6–10 hr cultures.

(A) A control neuron, patched but not stimulated, exhibited repulsive turning away from the netrin-1 source (5 μg/ml in the pipette). Images depict the growth cone immediately (0 min), 5 min, and 60 min after achieving the whole-cell recording condition. Bar = 20 μm. Note that the growth cone exhibited slight patching-induced retraction and subsequent resumption of its motility and migration away from the pipette (60 min). Traces on the right depict trajectories of neurite extension for all five cases. Bar = 10 μm. Inserts show the recorded membrane potentials during the experiment.

(B) Similar to that in (A), except a series of action potentials (6 pulses at 2 Hz) was initiated in the soma by injection of depolarizing currents through the whole-cell pipette (see inset). A sample trace of the action potential is shown on the right. Scales: 40 mV, 5 ms.

same gradient caused attractive turning of growth cones in 16–20 hr cultures (Figure 1A). In contrast, when 6–10 hr cultures were electrically stimulated (10 pulses at 2 Hz) prior to the application of the netrin-1 gradient, these young neurons exhibited attractive turning responses (Figure 1E). Thus, electrical activity had converted the repulsive effect of netrin-1 on these young neurons to attraction.

The electrical stimulation applied to the culture induced a transient depolarization of the neuronal membrane, as indicated by the twitch contraction of nerve-contacted myocytes in these cultures and by direct whole-cell recording of the neuronal membrane potential during the stimulation. That action potentials induced in the soma are sufficient to alter the turning responses of the growth cone was further tested by direct electrical stimulation of the neuron (in 6–10 hr in culture) at the soma with a perforated-patch recording electrode. In most cases, the seal formation and perforation of the membrane patch resulted in retraction of the growth cone, with a disappearance of filopodia (Figures 2A and 2C). However, within 10–20 min, some growth cones recovered their motility and resumed active filopodial protrusion. Control neurons (in 6–10 hr in culture) under perforated patch recording in the absence of induced action potentials showed repulsive turning away from the netrin-1 source (5 μg/ml in the pipette), with a mean turning angle of $-19.0° \pm 7.7°$ (SEM, n = 5; Figure 2B). In contrast, when a train of six action potentials (at 2 Hz) was initiated at the soma by injection of depolarizing current pulses, the growth cone of young neurons exhibited attractive turning toward the netrin-1 source (Figures 2C and 2D), with a mean turning angle of $31.6° \pm 10.3°$ (SEM, n = 5). Furthermore, we noted that the resumption of filopodial motility was greatly enhanced by electrical stimulation.

Modulation of Responses Induced by rMAG and Myelin

These cultured *Xenopus* neurons are also sensitive to other guidance factors, including MAG (Song et al., 1998), a component of the myelin membrane that inhibits axon regeneration (McKerracher et al., 1994). We found that the repulsive turning responses of the growth cone (16–20 hr cultures) in a gradient of soluble recombinant MAG (rMAG; 150 μg/ml in the pipette) was converted to attraction by a brief period of electrical stimulation (10 pulses at 2 Hz) (Figure 3A). The electrical stimulation used in this study had no obvious deleterious effect on these *Xenopus* neurons. In addition to the population studies described previously in this article, we examined the growth cone–turning behavior of the same neuron before and after the electrical stimulation. In 5/6 neurons, we found that the growth cone exhibited repulsive turning away from the source of rMAG prior to the stimulation and attractive turning immediately following the stimulation (Figures 3B and 3C). Similar "dual"-turning

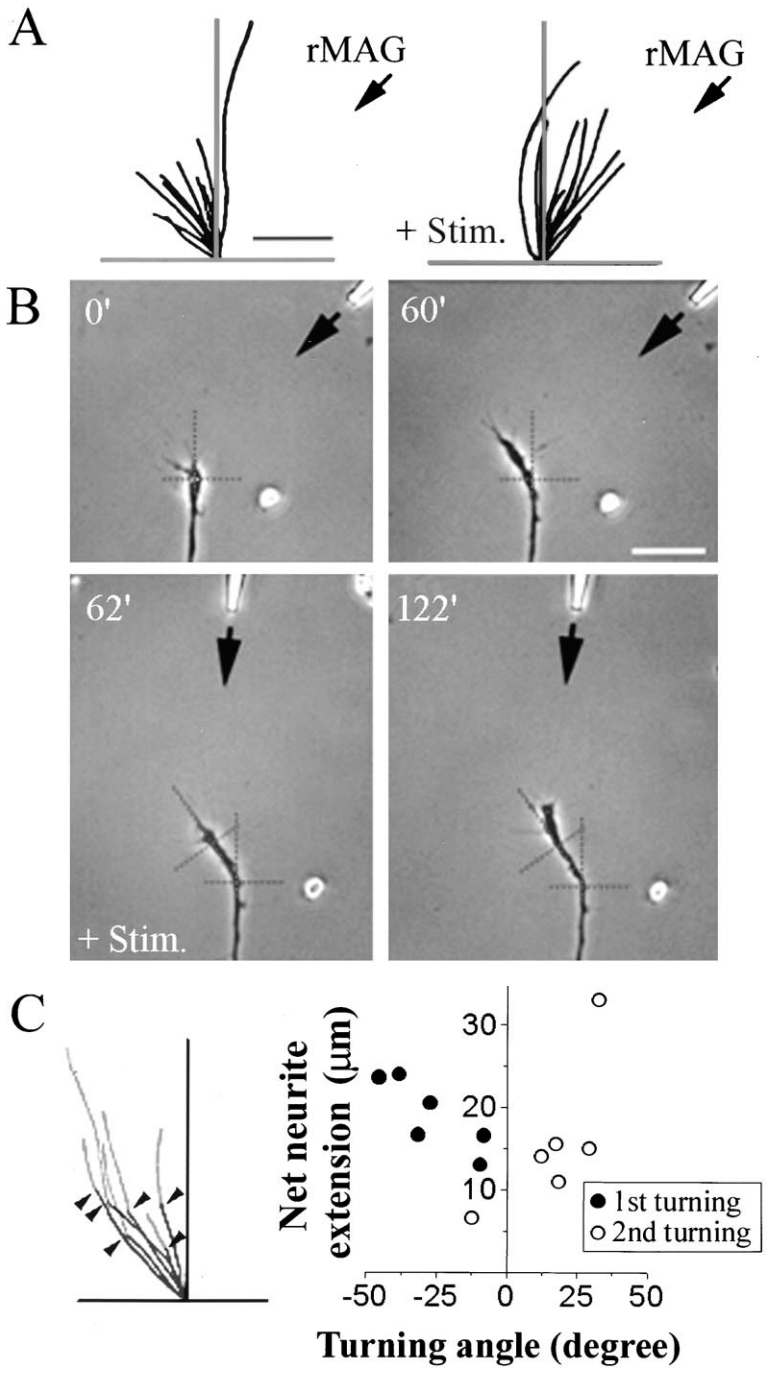

Figure 3. Modulatory Effects of Electrical Stimulation on Turning Responses Induced by Gradients of rMAG

(A) Trajectory tracings on the top depict the neurite growth induced by a rMAG gradient (150 μg/ml in the pipette) without (left) and with pre-exposure to field stimulation (same as in Figure 1C). The arrows indicate the direction of the rMAG gradient. Bar = 10 μm.

(B) Dual turning experiments (in 16–20 hr cultures). A growth cone was exposed to a rMAG gradient (150 μg/ml in the pipette) for 1 hr and was then electrically stimulated for 3 s (at 2 Hz) before further exposure (at 62′) to the same gradient for another hour. The pipette was repositioned to a site 100 μm away and oriented 45° with respect to the new neurite direction at 62′. The dashed lines depict the coordinates for pipette positioning at the beginning of the 1st and 2nd hr, respectively. Bar = 30 μm.

(C) Summary of dual turning experiments. Superimposed traces depict the trajectory of neurite extension of all six neurons examined in the manner described in (B). Arrows mark the end of the 1st hr. The scale is the same as in (A). Shown on the right is the scatter plot of net neurite extension and turning angles of the growth cone at the end of the 1st (filled symbols) and 2nd hr (open symbols).

assays were also performed for netrin-1-induced responses in young neurons (6–10 hr cultures), which showed conversion from repulsion to attraction in 3/3 neurons examined (data not shown).

In addition to MAG, which is only one of the inhibitory factors associated with myelin (Schwab et al., 1993), we examined the effect of a gradient of a suspension of myelin membrane fragments (25 μg/ml in the pipette) (McKerracher et al. 1994) on growth cone turning. As shown in Figure 4, growth cones of these *Xenopus* neurons exhibited repulsive turning in the myelin gradient. The repulsion was converted to attraction by pre-exposure of these neurons to the electrical stimulation (10 pulses at 2 Hz). This suggests that neuronal responses to other inhibitory factors associated with the myelin membrane can also be modulated in a similar manner as that of MAG-induced responses. Furthermore, as shown in Figure 4, the repulsive turning in the myelin gradient was converted to attraction by bath application of the cAMP analog Sp-cAMPS (Rothermel and Parker Botelho, 1988).

Dependence on the Number and Pattern of Electrical Stimulation

The effectiveness of electrical stimulation in modulating the turning response in a MAG gradient was further

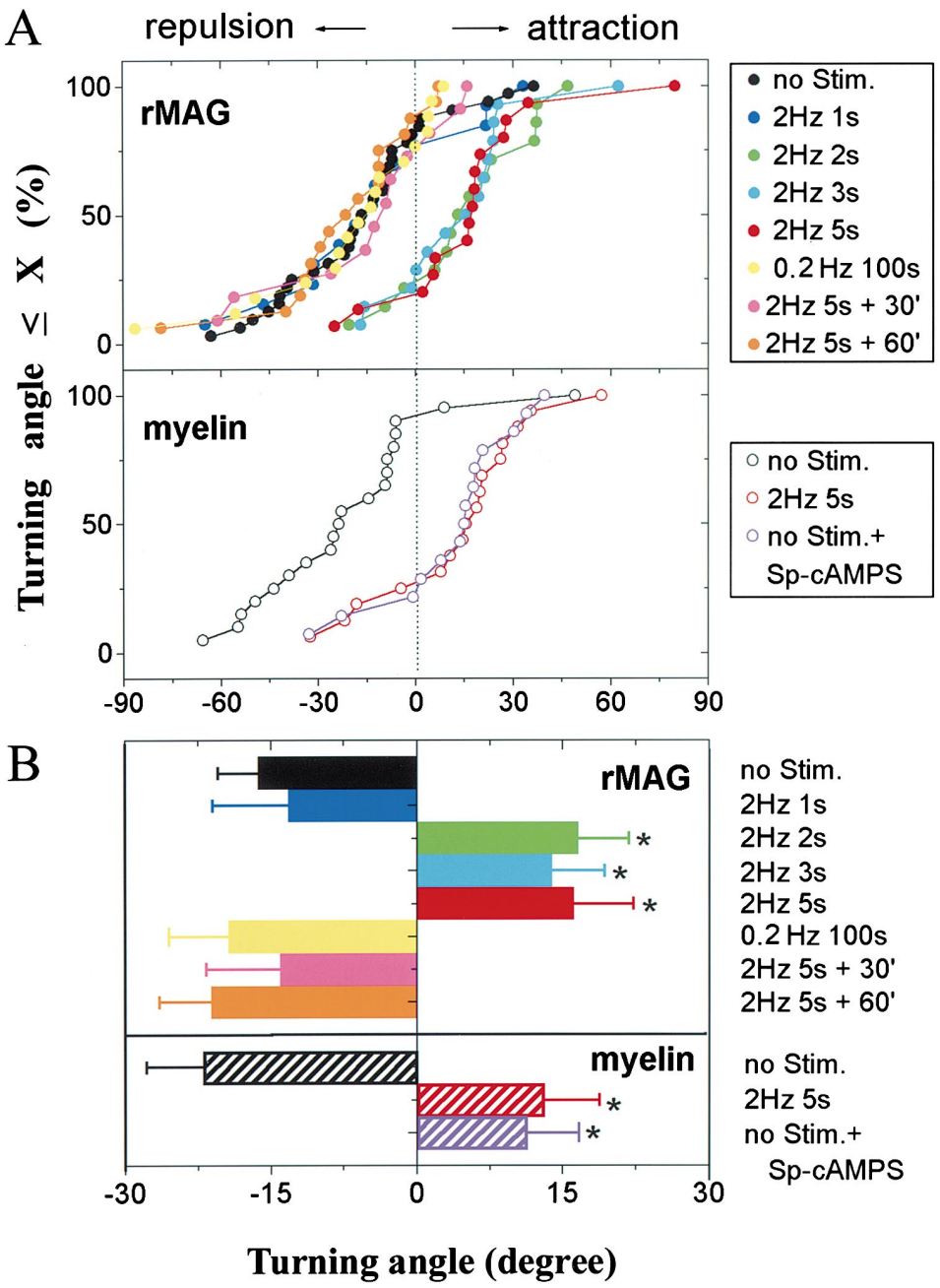

Figure 4. Turning of Growth Cones in Gradients of rMAG or Myelin Fragments—Dependence on the Number and Pattern of Stimuli

(A) Distribution of turning angles. For each experimental condition, angular positions of all growth cones at the end of a 1 hr exposure to gradients of rMAG (150 μg/ml in the pipette) or myelin fragments (25 μg/ml in the pipette) are shown in the cumulative percentage plot. The percent value refers to the percentage of growth cones with angular position ≤ a given angle. For the rMAG gradient, data shown are turning observed for control neurons not exposed to electrical stimulation and neurons treated with electrical stimuli of different numbers of pulses at different frequencies (3 V/cm, 2 ms) in 16–20 hr cultures. Data shown with "+30" or "+60" refer to experiments carried out 30 or 60 min after the stimulation. Data for the myelin fragment gradient were collected from 16–20 hr cultures for control neurons and neurons treated with electrical stimulation (same as in Figure 1C) or with bath application of a cAMP analogue, Sp-cAMPS (20 μM).

(B) The average turning angles for experiments shown in (A), coded by the same colors. Results that show significant differences from the control group (exposed to the gradient in the absence of stimulation) are marked ($p < 0.01$, Kolmogorov-Smirnov test).

examined by applying different numbers of electrical pulses at different frequencies. As shown in Figures 4A and 4B, a significant conversion of the turning response from repulsion to attraction was induced by a gradient of rMAG after only 4 pulses of stimulation at 2 Hz. On the other hand, low-frequency electrical stimulation (10 pulses at 0.2 Hz) was ineffective in modulating the turning response to rMAG (Figure 4), suggesting that the effect of electrical stimulation may be pattern dependent. Moreover, the effects of electrical stimulation appeared to persist for a period of less than 30 min, and the turning response to rMAG became repulsive when

tested 30 and 60 min after the electrical stimulation (Figure 4).

The Effects of Electrical Stimulation Require Ca^{2+} Influx

How does electrical activity alter the turning response of the growth cone? An immediate consequence of membrane depolarization is the opening of voltage-dependent Ca^{2+} channels and an influx of Ca^{2+}. When extracellular Ca^{2+} was removed during electrical stimulation, and then the turning assay was done in normal saline, we observed no significant effect of electrical stimulation on the subsequent turning behavior induced by netrin-1 or rMAG gradients (Figure 5). Thus, Ca^{2+} influx during stimulation appears to be necessary for the modulatory effect of the electrical activity. Whether our standard stimulation protocol can induce Ca^{2+} elevation in these neurons was further examined by imaging cytosolic Ca^{2+} at the growth cone, using the Ca^{2+} sensitive fluorescence dye Oregon Green BAPTA-dextran (Hong et al., 2000). As shown in Figures 6A and 6B, a brief train of electrical stimuli (10 pulses at 2 Hz) led to an immediate transient elevation of Ca^{2+} in these neurons, followed by a more persistent low-level plateau phase of Ca^{2+} elevation (Figure 6B), whereas neurons stimulated with 2 pulses showed a smaller Ca^{2+} transient of shorter duration (Figures 6A and 6C). Because the effect of the stimulation appears to persist for a period longer than that of Ca^{2+} elevation (data not shown), the Ca^{2+} signal must have induced a more persistent change in its downstream effectors in order to sustain the stimulation effect. It is known that electrical activity can suppress neurite elongation and growth cone motility via Ca^{2+} elevation (Cohan and Kater, 1986; Gu and Spitzer, 1995). Here we show that the Ca^{2+} signal, induced by a brief period of electrical stimulation, is also capable of modulating the path-finding behavior of the growth cone. Apparently, different patterns of electrical stimulation and the resultant Ca^{2+} elevation are responsible for different aspects of growth cone motility. This is consistent with the observation that a gradient of Ca^{2+} elevation in the growth cone may induce either attractive or repulsive turning, depending on the spatiotemporal profiles of Ca^{2+} signals (Hong et al., 2000; Zheng, 2000).

The Effects Are Mediated through Elevation of cAMP

Electrical activity is known to increase cAMP levels in neurons (Hempel et al., 1996), presumably through activation of Ca^{2+}-dependent adenylate cyclase (Xia and Storm, 1997), and the direction of growth cone turning induced by many guidance factors can be modulated by pharmacological manipulation of the cytosolic level of cAMP (Song et al., 1997; Ming et al., 1997b, 1999; Song and Poo, 1999). Thus, we examined the role of cAMP-dependent pathways in the electrical stimulation–induced modulation of growth cone behavior. When an inhibitor of adenylate cyclase, SQ22536 (Talpain et al., 1995), was added to the culture during electrical stimulation (and washed away before the turning assay), the effect of stimulation on the conversion of rMAG-induced turning behavior was totally abolished (Figure 5). Furthermore, bath application of a competitive analog of cAMP, Rp-cAMPS (20 μM), or a specific inhibitor for protein kinase A (PKA), KT5720 (200 nM) (Kase et al., 1987), during and after the electrical stimulation (4 pulses at 2 Hz) had a similar blocking effect for the conversion of rMAG-induced turning (Figure 5). Similarly, no significant effect of electrical stimulation on the turning behavior in a gradient netrin-1 was observed in the presence of Rp-cAMPS (20 μM). These results are consistent with the idea that electrical stimulation elevates cAMP through activation of Ca^{2+}-dependent adenylate cyclase (Xia and Storm, 1997), which, in turn, is sufficient to modulate the guidance behavior of the growth cone.

Modulation of Semaphorin 3A-Induced Responses

These growth cones are also known to exhibit repulsive turning responses in a gradient of Semaphorin 3A (Sema3A) in a manner that is independent of the cytosolic level of cAMP (Song et al., 1998). Does electrical stimulation convert Sema3A-induced repulsion into attraction? Surprisingly, we found that the same electrical stimulation greatly enhanced the effect of Sema3A in inducing the collapse of the growth cone in 16–20 hr cultures. Following stimulation, a gradient of Sema3A (50 μg/ml in the pipette) that normally induces repulsive turning of the growth cone resulted in growth cone collapse in nearly all neurons examined (Figures 7A and 7B). When the electrical stimulation was applied in the absence of extracellular Ca^{2+} (turning tested in normal Ca^{2+}) or when both the stimulation and turning were tested in the presence of Sp-cGMPS (10 μM) (Butt et al., 1990), a membrane-permeable analog of cGMP, we observed no collapsing effect of the Sema3A gradient. Furthermore, when the dose of Sema3A in the pipette was reduced to a level (5 μg/ml in the pipette) below the effective concentration for the induction of repulsive turning responses (Song et al., 1998) (Figure 7C), electrical stimulation resulted in significant repulsion in the gradient. Thus, electrical activity modulates Sema3A-induced guidance behavior by enhancing the repulsive and collapsing activity of Sema3A, a situation opposite to that found for netrin-1, MAG, and myelin membrane fragments. Although Sema3A-induced growth cone repulsion in these cultures is not affected by lowering extracellular Ca^{2+} (Song et al., 1998), we found that the enhancement of the Sema3A-induced collapse by electrical stimulation required the presence of extracellular Ca^{2+} during stimulation, consistent with the requirement of depolarization-induced Ca^{2+} influx. Furthermore, the addition of Sp-cGMPS (10 μM) to the culture during and after electrical stimulation resulted in attractive turning of the growth cone in a gradient of a high dose (50 μg/ml in the pipette) of Sema3A (Figure 7C). These results, together with our previous finding on the cGMP-dependent modulation of Sema3A-induced turning (Song et al., 1998), suggest that electrical stimulation may result in a reduction of the cytosolic level of cGMP, an effect that can be masked by the addition of the cGMP analog. Consistent with this interpretation, we observed that the effect of electrical stimulation can be partially mimicked by Rp-cGMPS (Butt et al., 1990), a competitive analog of cGMP: Growth cones exhibited largely repulsive turning in the low-dose Sema3A gradient in the presence

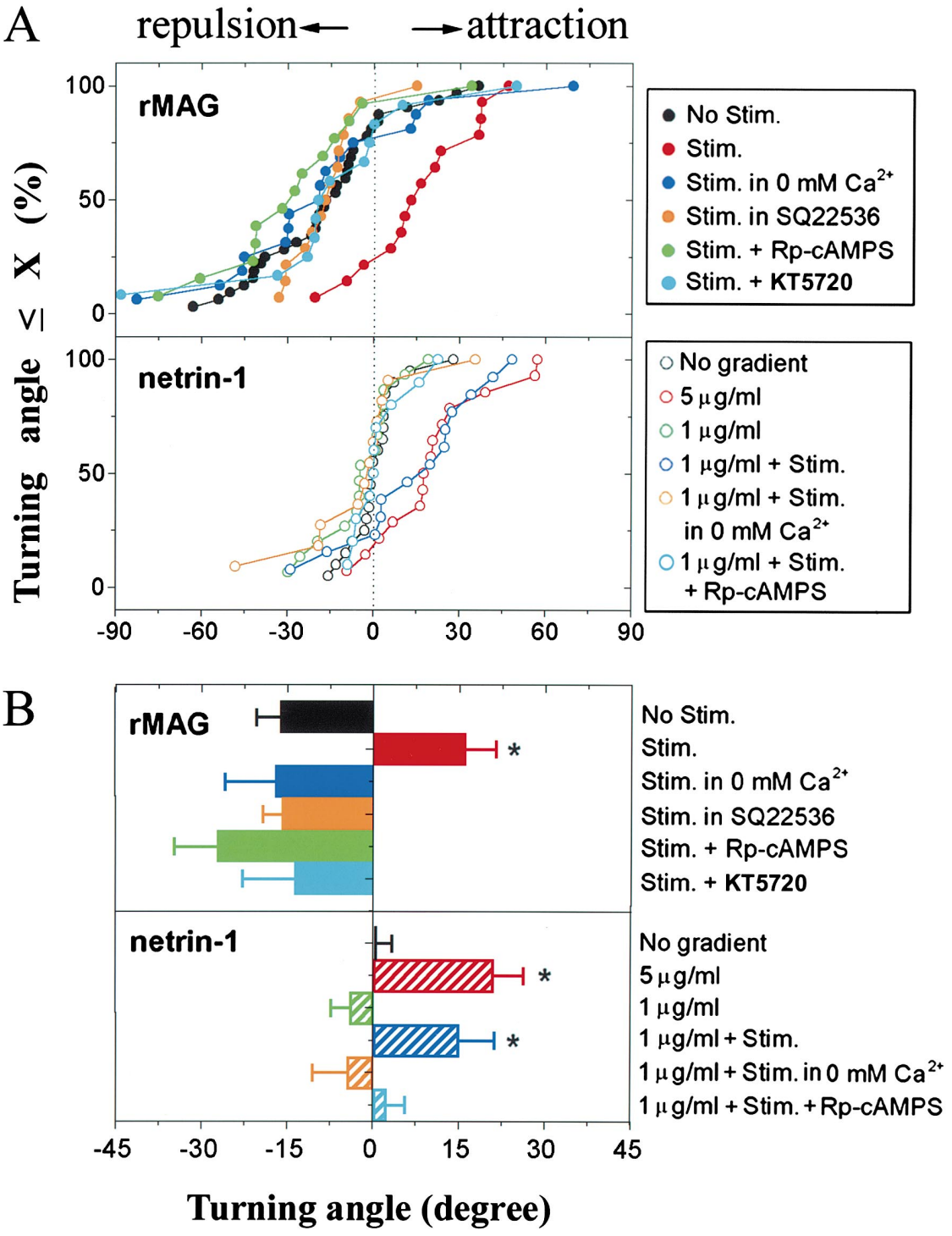

Figure 5. Effects of Stimulation Require Ca^{2+} and Are Mediated through cAMP Signaling

(A) Distribution of turning angles in a gradient of rMAG (150 μg/ml in the pipette) or netrin-1 (1 or 5 μg/ml in the pipette) for neurons without or with electrical stimulation (4 pulses at 2Hz for rMAG experiments and 10 pulses at 2 Hz for netrin-1 experiments) in normal saline or saline containing 0 mM Ca^{2+}, Rp-cAMPS (20 μM), the adenylate cyclase inhibitor SQ22536 (2 μM), or the PKA inhibitor KT5720 (200 nM).

(B) The average turning angles for experiments shown in (A), coded by the same colors. Results that show significant differences from the control group (exposed to the gradient in the absence of stimulation for rMAG experiments or in the absence of gradient and stimulation for netrin-1 experiments) are marked (*$p < 0.01$, Kolmogorov-Smirnov test).

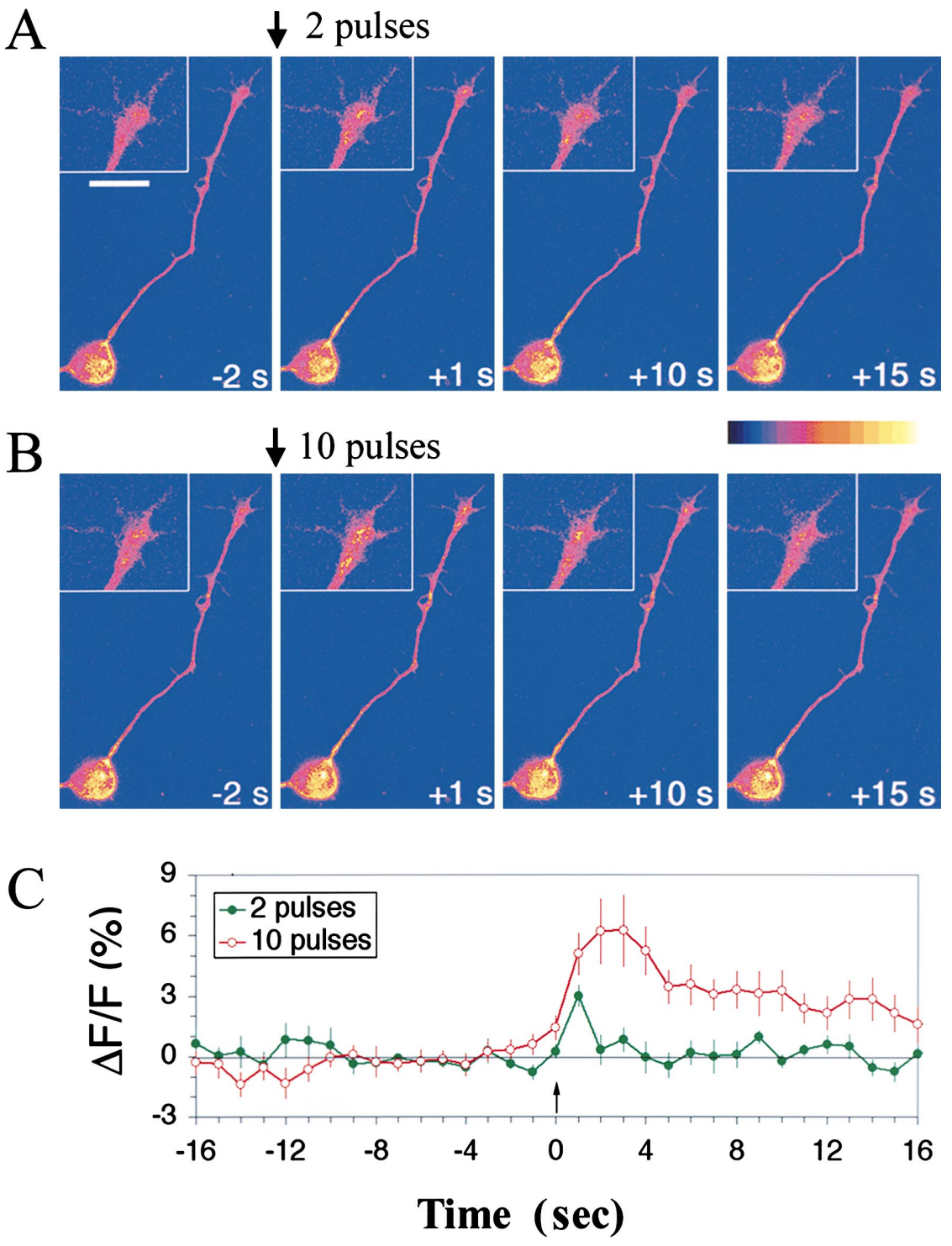

Figure 6. Elevation of Ca^{2+} in the Growth Cone Induced by Electrical Stimulation

(A and B) Fluorescence images of the growth cone of a *Xenopus* spinal neuron, which was microinjected with the Ca^{2+} indicator Oregon Green BAPTA-dextran. Bar = 20 μm. Confocal images were acquired at different times before (−) and after (+) electrical stimulation ([A], 2 pulses at 2 Hz; [B], 10 pulses at 2 Hz). The intensity of the fluorescence is coded by pseudo colors in linear scale shown on the right, with white representing the highest intensity.

(C) Normalized changes in Ca^{2+} levels as revealed by percentage changes in Oregon Green BAPTA-dextran fluorescence (ΔF/F ± SEM) in 6–10 growth cones. The fluorescence was normalized to the average value observed 30 s prior to the onset of the electrical stimulation, which is marked with an arrow.

of Rp-cGMPS (20 μM) without electrical stimulation (Figure 7C).

Discussion

In this study, we have examined the effects of brief electrical stimulation on the turning responses of *Xenopus* growth cones induced by four different types of guidance cues: netrin-1, MAG, myelin membrane fragments, and Sema3A. The attractive and repulsive actions of netrin-1, MAG, and Sema3A on these growth cones have been reported previously (Ming et al., 1997b; Song et al., 1998). Here we found that the effect of netrin-1 on *Xenopus* spinal neurons depends on the age of the culture. Neurons in 6–10 hr cultures exhibit repulsive turning of growth cones in a netrin-1 gradient, whereas the same gradient causes attractive turning in 16–20 hr cultures. Similarly, these growth cones exhibit collapsing or repulsive responses to BDNF in young but not old cultures (Wang and Zheng, 1998). The latter appears to result from a low endogenous level of cAMP in young neurons because application of db-cAMP is sufficient to prevent this collapsing effect (Wang and Zheng, 1998). These growth cones also exhibit marked attractive turning toward BDNF in older cultures (Ming et al., 1997a; Song et al., 1997). This is consistent with our observation that electrical stimulation converts the repulsive turning to attractive turning of these growth cones in a netrin-1 gradient, presumably by elevating the endogenous level of cAMP. Furthermore, pharmacological elevation of endogenous cAMP is known to increase the sensitivity of these growth cones toward gradients of low concentrations of attractants. For example, the attractive turning of growth cones induced by a gradient of BDNF was enhanced by forskolin (Song et al., 1997), a drug that elevates the endogenous production of cAMP. This is again consistent with our observation that electrical stimulation enhances the netrin-1-induced attractive turning responses in 16–20 hr cultures.

Previous studies have delineated two groups of guidance cues in terms of the modulatory effects of cyclic nucleotides on the turning behavior of the growth cone (reviewed by Song and Poo, 1999). The turning responses induced by group I cues, which include netrin-1, BDNF, and MAG, depend on the cytosolic level of cAMP, whereas those induced by group II cues, including Sema3A and neurotrophin-3, depend on the cGMP level. Increasing the concentration of the cyclic nucleotide favors attraction, whereas lowering its concentration favors repulsion. Myelin membrane fragments are known to contain several types of inhibitory factors for axon growth (Schwab et al., 1993). In the present study, we found that the growth cone–turning response induced by a gradient of myelin fragments can be modulated by cAMP-dependent activities (Figure 4), and both repulsive and attractive responses (after bath application of Sp-cAMPS) were abolished when extracellular Ca^{2+} was reduced to 1 μM (data not shown), similar to those found for group I guidance cues. Thus, most of the myelin-associated inhibitory factors are likely to share similar cytoplasmic signaling mechanisms as other group I cues (Song and Poo, 1999). Surprisingly, electrical stimulation has opposite modulatory effects on these two groups of cues, promoting attractive actions of group I cues and enhancing repulsive actions of group II cues. These effects are consistent with the idea that electrical stimulation increases the level of cAMP and decreases that of cGMP. Although there is evidence that electrical stimulation can activate adenylate cyclase to increase cAMP production (Hempel et al., 1996; Xia and Storm, 1997), it is not clear whether activities of guanylate cyclase or cGMP phosphodiesterase can be affected by electrical stimulation.

Our finding that only a few pulses of electrical stimulation can trigger marked alteration in the turning behavior suggests that cytoplasmic signaling mechanisms in the growth cone are highly sensitive to physiological activities of developing neurons. Moreover, the same number of stimuli applied at a low frequency of 0.2 Hz was without effect, suggesting that the mechanism is sensitive to the pattern of stimuli. Developing neurons may receive synaptic inputs at their dendrites and become electrically active, whereas their axons are still in the process of path finding (Milner and Landmesser, 1999; O'Donovan, 1999). In the developing chick spinal cord, motor neurons exhibit regular bursts of activity from early embryonic stages before their growing axons reach the target muscles (Milner and Landmesser, 1999). As opposite turning behaviors can be triggered following the presence of activity, electrical activity may play an instructive role in the path finding of growing axons. Although blocking synaptic transmission by mutation of the synaptic protein Munc 18 did not produce gross developmental defects of the nervous system (Verhage et al., 2000), spontaneous neuronal spiking may still be required for the path finding and initial targeting of axons. This was exemplified by the findings of aberrant patterns of axon targeting in the developing cortex following activity blockade with tetrodotoxin (Catalano and Shatz, 1998; Dantzker and Callaway, 1998). The role of activity was also shown for the path finding of some olfactory neurons. In mice that are deficient in a cyclic nucleotide-gated channel subunit, which is responsible for odorant-evoked neuronal activity, projection of olfactory sensory axons to the glomeruli in the olfactory bulb was affected for axons expressing one odorant receptor but not another (Lin et al., 2000; Zheng et al., 2000), suggesting that the guidance behavior of different neuronal populations may exhibit differential sensitivity to the regulation by electrical activity. One potential factor in such differential sensitivity is the level of endogenous cyclic nucleotides, upon which the activity exerts its modulatory effect.

Prolonged application of small DC currents that generate fields in the order of 0.1 to 1 V/cm causes orientation of neurite outgrowth from cultured explants (Marsh and Beams, 1946; Sisken and Smith, 1975; Jaffe and Poo, 1979) and "galvanotropic" turning of the growth cone of *Xenopus* spinal neurons (Hinkle et al., 1981; Patel and Poo, 1982, 1984). Application of DC currents by implanted batteries also enhances nerve regeneration of severed spinal cord (Borgens et al., 1987) and optic nerve in vivo (Politis et al., 1998). Brief electrical stimulation was found to promote the speed and accuracy of motor axon regeneration (Al-Majed et al., 2000). The underlying mechanisms for these effects of electri-

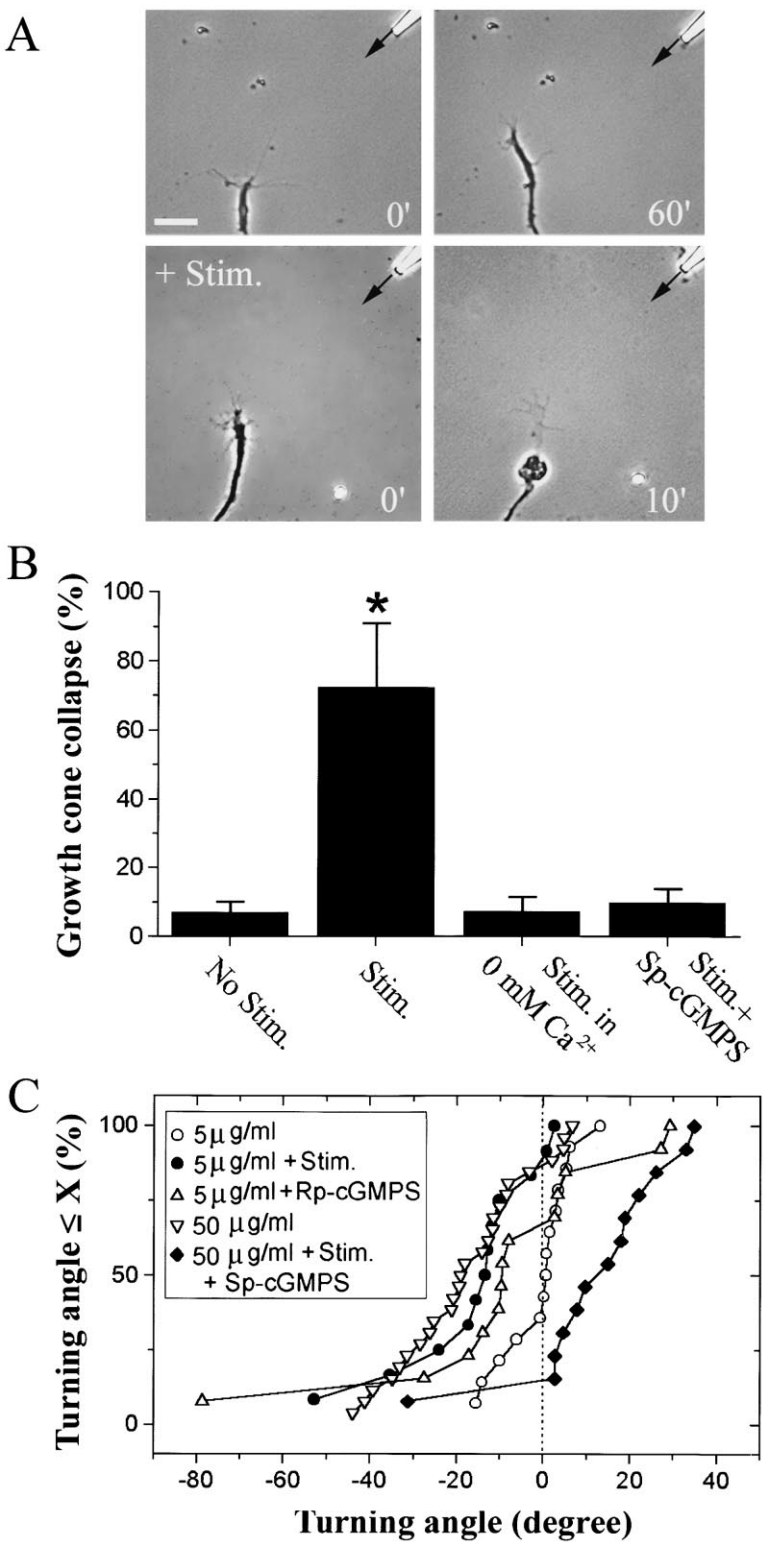

Figure 7. Modulatory Effects of Electrical Stimulation on Growth Cone Behavior Induced by Sema3A

(A) An example of stimulation-enhanced growth cone collapse. A gradient of a high dose of Sema3A (50 μg/ml in the pipette) induced a repulsive turning response of the growth cone in 16–20 hr cultures (top two images). The same gradient induced growth cone collapse of a neuron that was exposed to field stimulation (2 Hz for 5 s) prior to the onset of the gradient (bottom two images). Bar = 10 μm.

(B) Summary of the effects of field stimulation on growth cones in the presence of a Sema3A gradient as that described in (A). The percentage of growth cones that collapsed was shown for those not exposed to field stimulation ("No Stim."), exposed to stimulation ("Stim.") in the absence of extracellular Ca^{2+} (0 mM) or in the presence of Sp-cGMPS (10 μM). Results that show significant differences from the control group (exposed to the gradient in the absence of stimulation) are marked (*$p < 0.01$, Kruskal-Wallis test).

(C) Distribution of turning angles for experiments with gradients of Sema3A of a low dose (5 μg/ml) or high dose (50 μg/ml), with and without field stimulation, and in the presence of Rp-cGMPS (20 μM) or Sp-cGMPS (10 μM).

cal stimulation remain largely obscure. Our findings here suggest a plausible mechanism for the enhancement of nerve regeneration by electrical stimulation in vivo, that is, a conversion of the action of myelin-associated inhibitory factors from repulsion to attraction. Prolonged application of DC currents within the tissue creates deleterious side effects caused by accumulation of electrode products. The effectiveness of brief pulsatile stimuli in modulating growth cone behavior suggests that intermittent electrical stimulation may serve as an effective and safe therapeutic method for promoting nerve regeneration after injury.

Experimental Procedures

Culture Preparation

Cultures of *Xenopus* spinal neurons were prepared from the neural tube tissue of 1-day-old *Xenopus* embryos on glass coverslips by methods previously described (Tabti et al., 1998). The culture medium consisted of 50% (v/v) Leibovitz medium (GIBCO, Gaithersburg, MD), 1% (v/v) fetal bovine serum (HyClone, Logan, UT), and 49% (v/v) Ringer's solution (in mM: 115 NaCl, 2 $CaCl_2$, 2.5 KCl, and 10 HEPES [pH 7.4]). The cultures were used between 6–10 or 16–20 hr after plating at room temperature (20°C–22°C) for each experiment.

Production of Chemical Gradients and Growth Cone–Turning Assay

Microscopic gradients of chemicals and diffusible factors were produced by methods previously described (Ming et al., 1997a, 1997b; Song et al., 1997, 1998). Theoretical analysis (Lohof et al., 1992) and direct measurements of the gradient using fluorescent dyes (Zheng et al., 1994) have shown that at a distance of 100 μm from the pipette tip, the concentration gradient across the growth cone (typical width of 10 μm) is in the range of 5%–10%, and the average concentration is approximately 10^3-fold lower at the growth cone than that in the pipette. Different concentrations of the molecules in the pipette result in different average extracellular concentrations at the growth cone, but with the same gradient. To assay growth cone turning, the pipette tip was placed 100 μm away from the center of the growth cone of an isolated neuron and at an angle of 45° with respect to the initial direction of neurite extension (indicated by the last 10 μm segment of the neurite). The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1 hr period. Microscopic images of neurites were captured with a CCD camera (Toshiba IK-541RA) attached to a phase contrast microscope (Nikon TMS) into a computer for later analysis using NIH Image programs. To determine the total length of neurite extension, the entire trajectory of the neurite at the end of the 1 hr period was measured with a digitizer. Only those growth cones with net extension of >5 μm over the 1 hr period were included for analysis of turning angles. All experiments were carried out at room temperature in modified Ringer's solution (in mM: NaCl 140, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10 [pH 7.40]). Calcium-free saline contained (in mM) the following: NaCl 140, $MgCl_2$ 1, KCl 5, EGTA 4, HEPES 10 (pH 7.40). For pharmacological treatments, chemicals (Calbiochem, La Jolla, CA) were added to the culture medium at least 20 min before and were present during the experiments or otherwise as described. Recombinant netrin-1, Sema3A, and rMAG were produced as previously described (Song et al., 1998). Myelin membrane fragments were prepared from bovine brain as previously described (McKerracher et al., 1994) and were resuspended in phosphate-buffered saline.

Electrical Stimulation and Electrophysiology

Field stimulation of the neuron was performed as previously described (Ryan and Smith, 1995). The culture was transferred into a recording chamber with two parallel silver chloride wires located close to the surface of the coverslip. Action potentials were stimulated by passing 2 ms bipolar current pulses (at 2 Hz), yielding an electric field of approximately 3 V/cm across the chamber. Cells were maintained in modified Ringer's solution at room temperature throughout the experiments. For direct electrical stimulation of neurons, whole-cell perforated patch recordings from the soma of *Xenopus* spinal neurons were performed using amphotericin B (Sigma, St. Louis, MO) for perforation (Horn and Marty, 1988). The micropipette resistance was in the range of 2–4 MΩ. The pipettes were tip filled with internal solution and then back filled with internal solution containing 200 μg/ml amphotericin B. The internal solution contained (in mM) the following: potassium gluconate 136.5, KCl 17.4, NaCl 9, $MgCl_2$ 1, HEPES 10, and EGTA 0.2 (pH 7.30). The neurons were held in current-clamp by patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA), and the membrane potentials were continuously monitored throughout each experiment. Signals were filtered at 5 kHz using amplifier circuitry, and data were acquired at 10 kHz with a computer and then analyzed using pClamp 6.0 software (Axon Instruments). Action potentials were triggered by injecting current pulses (1–2 ms; 1–1.5 nA) through the recording pipette at 2 Hz.

Calcium Imaging

Isolated *Xenopus* spinal neurons were microinjected at the soma with the fluorescence Ca^{2+} indicator Oregon Green 488 BAPTA-1 conjugated to 70 kDa dextran (Molecular Probes, Inc., Eugene, OR) and imaged with a BioRad confocal imaging system (MRC-1024ES) as described previously (Hong et al., 2000). Images, which were acquired every 0.5–1 s, were analyzed using NIH Image. The intensity of the fluorescence at the growth cone was measured over a fixed square area that covered the entire growth cone throughout the measurement period.

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