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Retinal Ganglion Cells Do Not Extend Axons by Default: Promotion by Neurotrophic Signaling and Electrical Activity

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Summary

We investigate the signaling mechanisms that induce retinal ganglion cell (RGC) axon elongation by asking whether surviving neurons extend axons by default. We show that bcl-2 overexpression is sufficient to keep purified RGCs alive in the absence of any glial or trophic support. The bcl-2-expressing RGCs do not extend axons or dendrites unless signaled to do so by single peptide trophic factors. Axon growth stimulated by peptide trophic factors is remarkably slow but is profoundly potentiated by physiological levels of electrical activity spontaneously generated within embryonic explants or mimicked on a multielectrode silicon chip. These findings demonstrate that these surviving neurons do not constitutively extend axons and provide insight into the signals that may be necessary to promote CNS regeneration.

Introduction

Although much recent attention has focused on the signaling mechanisms that promote the survival of developing neurons and guide the growth of their axons, we still know relatively little about the mechanisms that control axon elongation. Do surviving neurons extend axons constitutively? Or, instead, must axon elongation be specifically induced by extracellular signals? It has been difficult to address this question experimentally because the survival of nearly all animal cells depends on extracellular signaling (Raff, 1992). Thus in order to study axon growth by neurons in culture, neurotrophic signals must be added to the culture medium to maintain their survival. Under these conditions, the effects of trophic signaling on survival and axon growth become difficult to separate.

We have been studying the signaling mechanisms that control the survival, growth, and regeneration of developing retinal ganglion cells (RGCs), a type of CNS neuron. RGCs fail to extend their axons in the optic nerve after injury and, like most CNS neurons, die after being severed from their targets (Goldberg and Barres, 2000). Recently, evidence has shown that RGC survival can be promoted in culture and after optic nerve axotomy in response to a combination of peptide trophic factors together with simultaneous cAMP elevation, which promotes neurotrophic responsiveness (Shen et al., 1999). In culture, these signals promote both survival and the growth of RGC axons (Meyer-Franke et al., 1995). These observations raise the question of whether peptide trophic factors primarily promote cell survival, with surviving cells extending axons by default, or instead signal both survival and axon elongation.

A number of previous studies on axon growth by PNS neurons relate to this question. Local application of neurotrophins to a growth cone enhances the rate of growth cone extension in vitro (Letourneau, 1978) and in vivo (Tucker et al., 2001). Developing peripheral neurons that lack the pro-apoptotic protein Bax do not undergo apoptosis, and weakly elaborate axons in vitro in the absence of added neurotrophic support (Deckwerth et al., 1996). Glial or even autocrine neuronal factors released within these cultures, however, might well have induced the observed axon growth in these previous experiments (Acheson et al., 1995). Taken together, these previous findings show that PNS axon growth rate is enhanced by local stimulation of growth cones, but do not address whether surviving PNS or CNS neurons can constitutively extend axons in the absence of neurotrophins or other signals (Goldberg and Barres, 2000).

The role of electrical activity in regulating axon growth is also unclear. Electrical activity has recently been demonstrated to potentiate the ability of neurotrophins to promote the survival and dendritic growth of CNS neurons (Mao et al., 1999; McAllister et al., 1996; Meyer-Franke et al., 1995; Sretavan et al., 1988). These studies raise the question of whether electrical activity also promotes axon elongation. Electrical stimulation does not affect axon growth rates by motor neurons (Ming et al., 2001) but strongly slows axon growth by sensory neurons (Cohan and Kater, 1986). Until recently, it has been technically difficult to experimentally manipulate the level of electrical activity of CNS neurons, other than to chronically depolarize neurons or entirely block their activity. Recently, multielectrode silicon chips have allowed direct manipulation and recording of the temporal and spatial pattern of electrical activity of neurons in culture (Gross et al., 1993; Borkholder et al., 1997; Maher et al., 1999; Gramowski et al., 2000), and the use of neuron-silicon interfaces is rapidly gaining momentum in multiple areas of investigation (Fromherz and Stett, 1995; Service, 1999; Scholl et al., 2000; Jenkner et al., 2001).

In the present studies, we have investigated these questions taking advantage both of the ability of the anti-apoptotic protein bcl-2 to promote neuronal survival and of a new silicon chip method. Exogenous bcl-2

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overexpression increases survival of RGCs in vivo during normal development (Martinou et al., 1994) and after axotomy both postnatally (Bonfanti et al., 1996) and in the adult (Cenni et al., 1996). We show that bcl-2 overexpression is sufficient to keep highly purified RGCs alive in culture at clonal density in the absence of any glial or trophic support. The bcl-2-expressing RGCs do not extend axons or dendrites by default, or in response to extracellular matrix molecules such as laminin alone, but require signaling by single peptide trophic factors. Furthermore, we show that axon growth in response to these factors is greatly potentiated by physiological levels of electrical activity. These findings demonstrate that these surviving CNS neurons do not constitutively extend axons and provide insight into the nature of signals that may be necessary for successful promotion of axon regeneration after CNS injury.

Results

Effect of Bcl-2 Expression on Retinal Ganglion Cell Survival In Vitro

To find out whether RGCs overexpressing bcl-2 would survive in the total absence of exogenous trophic support, we cultured highly purified postnatal day 8 (P8) RGCs on poly-D-lysine (PDL) in defined, serum-free medium (Meyer-Franke et al., 1995) (see Experimental Procedures). Immediately after isolation, the purified RGCs were infected with a recombinant adenovirus encoding the human *bcl-2* gene (adeno-*bcl-2*) or a control adenovirus encoding green fluorescent protein (adeno-GFP; Ehrengruber et al., 1998). Exposure to these adenoviral vectors reliably infected most of the RGCs, as assessed by GFP expression or staining with an antibody that detected human, but not rat, bcl-2 (Figures 1A–1C). In infected RGCs, human bcl-2 immunoreactivity was detected in both the cell bodies and neurites of infected (Figure 1B) but not uninfected cells (Figure 1C). Bcl-2 expression was sustained at high levels 14 days after infection, the longest time point that we examined. Multiples of infection of about 400 reliably infected 50%–80% of the RGCs, based on human bcl-2 immunoreactivity.

We next determined the effects of bcl-2 expression on RGC survival. Adeno-bcl-2 but not adeno-GFP infection was sufficient to prevent apoptosis for at least 3 days in the majority of purified RGCs in serum-free culture, despite the absence of all neurotrophic support (Figure 1D). BDNF (brain-dervied neurotrophic factor), CNTF (ciliary-derived neurotrophic factor), insulin, and forskolin (Growth Medium) together promote the survival of at least 80% of RGCs for 3 days in vitro (Meyer-Franke et al., 1995). Interestingly, bcl-2 expression alone was sufficient to elicit similarly high survival, but was not able to increase survival rates over those seen with RGC growth media (Figure 1D). Furthermore, the survival effect of adeno-bcl-2 infection was dose dependent: as adeno-bcl-2 infection titers were raised, RGC survival rates progressively increased in both embryonic and postnatal RGCs (Figures 1E and 1F), and paralleled the increase in percent of RGCs exhibiting bcl-2 immunoreactivity seen at increasing infection titers (data not shown). Even when P8 RGCs were plated at clonal density ($<$5 cells/mm^2) to eliminate autocrine or paracrine trophic signaling, 50% of postnatal RGCs in bcl-2-infected cultures were alive after 7 days of culture. These experiments show that bcl-2 overexpression is sufficient to promote the survival of purified RGCs in culture in the absence of any added trophic support.

Effect of Bcl-2 Expression on Axon Growth

We next investigated whether bcl-2-expressing, surviving RGCs extended axons by default in the absence of exogenous peptide signals. In the absence of neurotrophic support, the vast majority of RGCs expressing bcl-2 did not extend axons or dendrites, even after 7 days in culture (Figures 2A and 2C). Despite the absence of neurotrophic factors, the RGCs retained a strikingly healthy appearance maintaining large, round somas with a diameter of about 10–20 μm, which did not atrophy significantly over the 7 day culture period (Figure 2A). In contrast, RGCs cultured in defined Growth Medium rapidly extended processes within 24 hr and continued to elongate these processes after this period (Figures 2B and 2C). Virtually all surviving RGCs extended axon and dendrites under these conditions, as assessed by morphology and by immunostaining with the typical markers MAP2 for dendrites and tau for axons, as previously shown (Meyer-Franke et al., 1995). Identical results were observed when embryonic E20 RGCs were used instead of P8 RGCs. These results demonstrate that surviving RGCs do not extend either axons or dendrites by default, but need to be specifically signaled to do so.

It is possible that RGCs are competent to extend axons acutely after axotomy but then lose this ability over time. We found, however, that although bcl-2-expressing RGCs cultured in minimal media without trophic support for 7 days did not grow any axons or dendrites, when we added growth media containing neurotrophic factors back to the medium after 7 days, neurite outgrowth recommenced (Figure 2D). Therefore, RGCs remain healthy and competent to extend axons well after axotomy, as long as their survival is maintained during the interim.

We also asked whether removal of neurotrophic factors from RGCs that had already regenerated their axons in vitro caused the axons to die. We observed that 98% of RGCs cultured for 3 days in Growth Medium and then switched to minimal medium lacking peptide trophic factors showed complete regression of their axons to the cell soma, although the neuron survived if it was expressing bcl-2. Thus, as was demonstrated for PNS neurons (Campenot, 1994), RGCs require ongoing signals to maintain their axons.

Effects of Peptide Trophic Factors on Axon Growth

We next investigated the minimal signals sufficient to induce axon growth. To minimize autocrine and paracrine signaling, bcl-2-expressing P8 RGCs were cultured at clonal density ($<$5 cells/mm^2). After 3 days, RGCs were labeled with the vital dye calcein, which is cleaved to a fluorescent substrate by cytoplasmic esterases. Calcein brightly labeled the axons and dendrites in addition to cell bodies (Figure 3A). We confirmed our morphologic identification of axons by immu-

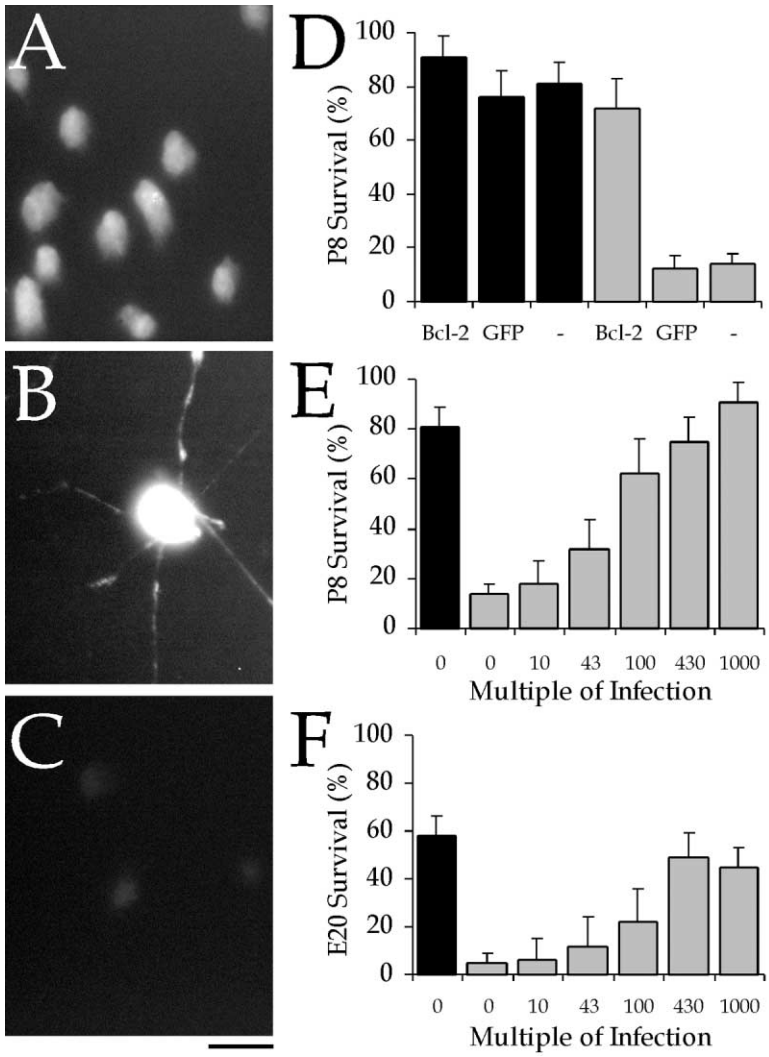

Figure 1. Bcl-2 Immunoreactivity and Survival of Rat Retinal Ganglion Cells

P8 RGCs were purified by immunopanning and infected with adeno-bcl-2. Human-specific bcl-2 immunoreactivity was detectable at 1 DIV (A) and at 3 DIV (B) but not in uninfected controls (C); scale bar 20 μm. (D–F) RGC survival was assayed by MTT assay after 3 days in defined media with (black bars) or without (gray bars) BDNF, CNTF, insulin, and forskolin. Postnatal day 8 (E) and embryonic day 20 (F) RGC survival increased in direct proportion to the amount of viral vector added.

nostaining with tau and MAP2 antibodies, and found that all presumptive axons with typical thin morphology stained with tau and were MAP2 negative, whereas presumptive dendrites with typical thicker morphology were MAP2 positive. Because developing and regenerating RGCs must extend a single axon over long distances through the optic nerve and tract, we measured the length of the longest axon on each neuron after 3 days in culture, although some RGCs extended more than one axon from their cell bodies.

We first asked whether single peptide trophic factors would be sufficient to induce axon growth. Only a very slow rate of axon growth could be elicited by BDNF (50 ng/ml) alone (Figure 3B) or by a large variety of other peptide trophic factors that we tested. Because we previously found that peptide trophic factors were insufficient to promote RGC survival unless the cells were signaled to be responsive by cAMP elevation, we next tested the effects of cAMP elevation. The adenylate cyclase activator, forskolin (5 μM), profoundly elevates cAMP in purified RGCs (Shen et al., 1999), but was not sufficient to induce axon growth on its own (Figure 3B). Similarly, the nondegradable cAMP analog CPT-cAMP (chlorophenylthio-cAMP) (200 μM) did not promote axon growth. However, RGC axon growth in response to BDNF or other peptide trophic factors was greatly potentiated by cAMP elevation, by nearly 7-fold (Figure 3B). This was not limited to a particular developmental window, as RGCs purified from embryonic E20 and adult P21 rats showed a similar dependence on cAMP elevation to enhance trophic responsiveness (data not shown). Thus peptide trophic factors signal RGCs to extend axons, but RGCs are poorly responsive to these peptides unless they are simultaneously signaled by cAMP elevation.

We next asked whether a neuron's axon growth response and survival response to the same peptide trophic signal showed the same dosage sensitivity. We investigated the relative ability of a single neurotrophin, BDNF, to elicit two separate biological functions in the same neurons, survival and axon growth. RGCs were cultured in the presence of forskolin and increasing concentrations of BDNF, in two conditions: after infection with adeno-bcl-2, to test axon growth independent of survival, or after infection with adeno-GFP, to test survival. The concentrations of BDNF that elicited half maxi-

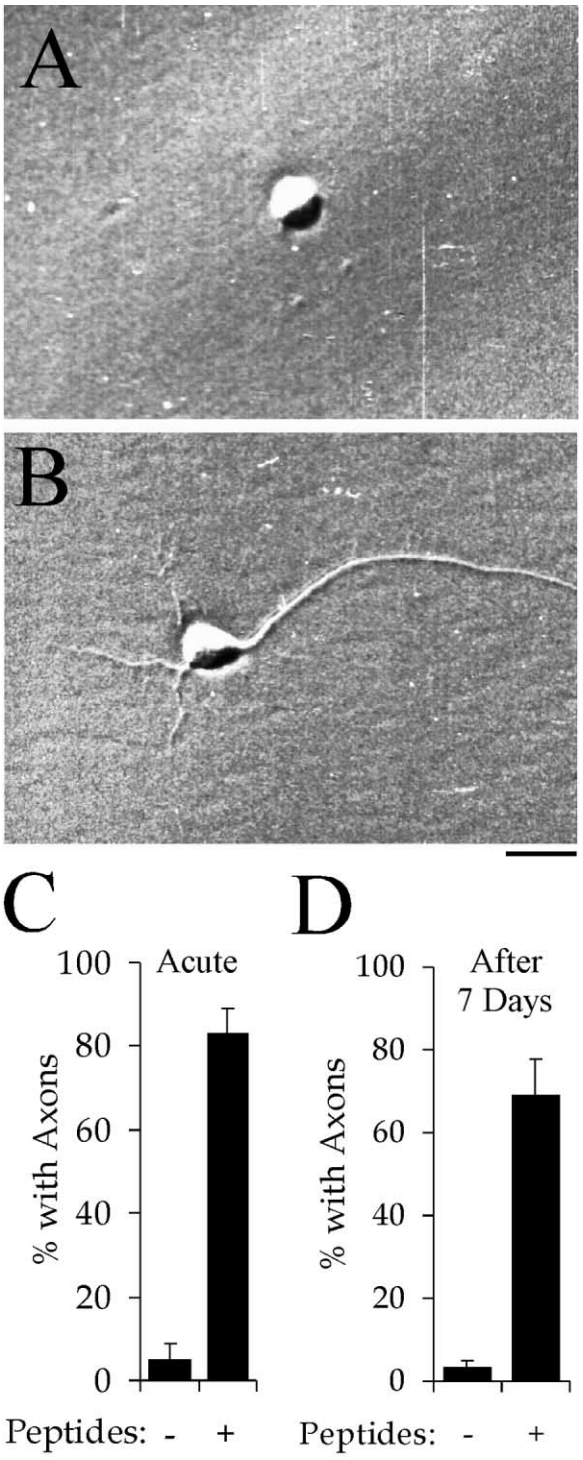

Figure 2. Axon Outgrowth in Adeno-bcl-2 Infected RGCs In Vitro

Purified RGCs were infected acutely with adeno-bcl-2 and cultured in the absence (A) or presence (B) of Growth Media; scale bar 30 μm. (C) Population data showing the percent of purified RGCs extending axons more than 1 cell body diameter (~15 μm; mean ± SEM). (D) Population data as in (C), for bcl-2-expressing RGCs first cultured 1 week in minimal media lacking peptide trophic factors and then returned to media with or without trophic factors as marked.

mal axon extension and survival were nearly identical at about 1–2 ng/ml (Figure 3C).

Many peptide factors other than BDNF were able to induce axon outgrowth in RGCs in the presence of cAMP elevation (Table 1). We found that every peptide trophic factor that supports RGC survival was also able to induce some axon growth (Meyer-Franke et al., 1995). For instance, CNTF and LIF (leukemia inhibitory factor) strongly promoted survival and also strongly promoted growth. We found that RGCs extended axons equally well in response to BDNF and CNTF or LIF (Table 1). IGF-1 (insulin-like growth factor 1), which supports RGC survival about half as well as BDNF or CNTF (Meyer-Franke et al., 1995), similarly induced approximately half the axon growth (Table 1). However, although the three peptide factors together elicit approximately additive survival rates (Meyer-Franke et al., 1995), the combination of BDNF, CNTF, and IGF-1 (or insulin) promoted a rate of growth faster than any of these alone, but much less than additive (Table 1). FGF-1 and -2, which have been widely reported to be strong axon growth stimulants (Lipton et al., 1988), were not very potent in stimulating axon growth in purified RGCs in the absence of other trophic signals (Table 1). FGFs (fibroblast growth factors) did potentiate both axon growth and survival in the presence of BDNF, however (data not shown). Interestingly, we found one factor, GDNF (glial-derived neurotrophic factor), which supported axon growth although it had no effect on RGC survival (Table 1). Thus, amongst a large variety of identified neurotrophic factors tested, BDNF and CNTF/LIF most strongly promoted axon growth, and combined together were even more effective.

Effects of Matrix Molecules on Axon Growth

Extracellular matrix molecules promote axon growth, but it is not known whether they are sufficient to induce growth on their own. To investigate this possibility, we next studied the ability of various extracellular matrix molecules to induce axon growth from purified P8 RGCs cultured at clonal density in the absence of added neurotrophic support. Extracellular matrix molecules used as a culture substrate, including laminin-1, merosin, collagen III, collagen IV, and the rich basement membrane matrix Matrigel, were each insufficient to induce significant axon growth on their own (Table 1). When these signals were added to the culture medium in soluble form, instead of being used as a substrate, they similarly did not induce axon growth (data not shown). When we examined the axon growth-promoting abilities of the cell-adhesion signaling molecules L1 or N-cadherin, or the axon guidance molecule netrin-1, there was similarly little effect (Table 1).

Laminin has previously been reported to enhance the ability of neurotrophins to stimulate neuronal survival and axon growth (Edgar, 1985). Although laminin-1 alone did not induce any axon growth, it significantly enhanced the rate of axonal growth stimulated by peptide trophic factors such as BDNF in P8 (Figure 3D) and E20 RGCs. For instance, laminin-1 enhanced BDNF-induced axon elongation by 2-fold. This effect was not significantly reduced in the presence of Rp-cAMP, an inhibitor of protein kinase A (data not shown). This potentiation

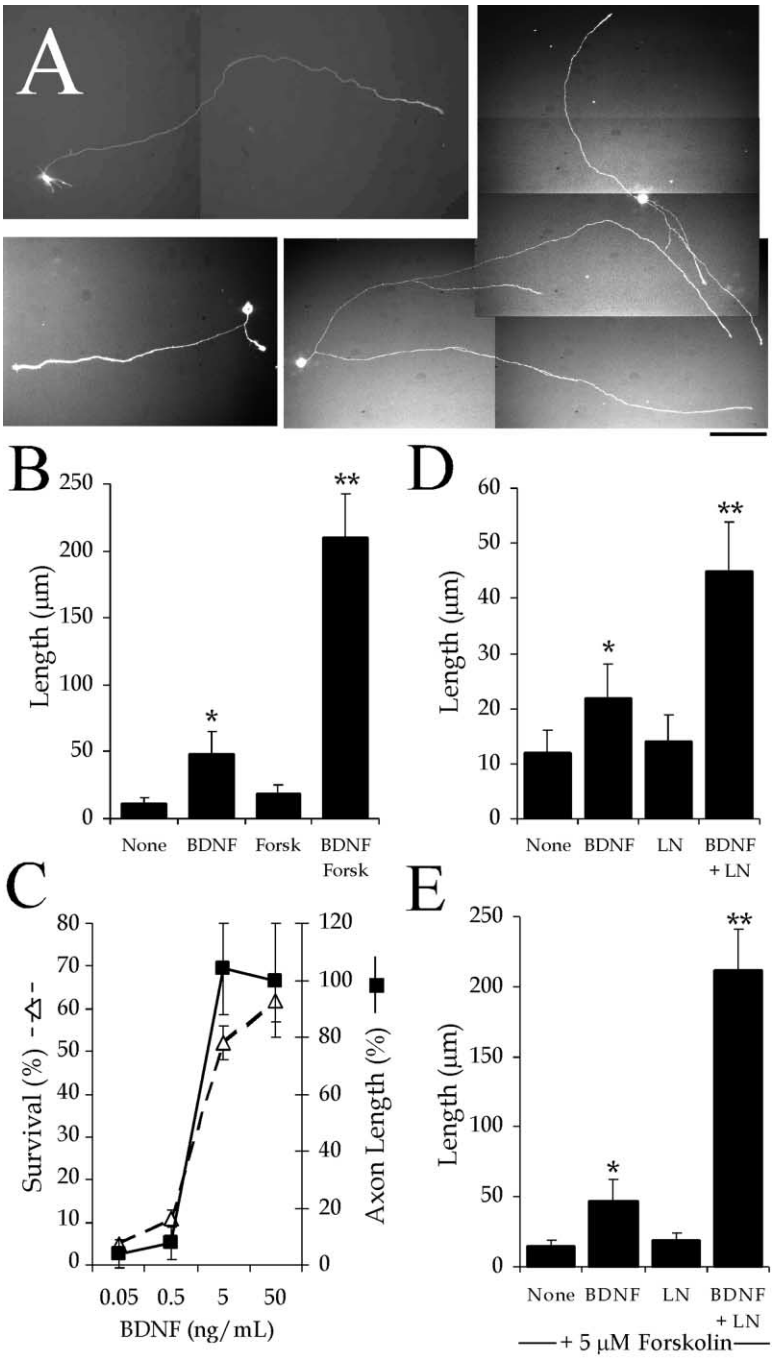

Figure 3. Quantitation of Axon Growth in Response to BDNF

(A) RGCs labeled with calcein exhibited bright fluorescence along their neurites; Scale bar, 100 μm. (B) RGCs were purified, infected with adeno-bcl-2, and cultured on laminin for 3 days at clonal density (<5 cells/mm^2) with BDNF (50 ng/ml) and forskolin (5 μM) as indicated. The longest axon on each RGC was quantitated (mean ± SEM). (C) RGCs were infected with adeno-bcl-2 to measure axon growth independent of survival, or with adeno-GFP to measure survival using the MTT assay. RGCs were cultured in forskolin (5 μM) and increasing concentrations of BDNF as indicated. Axon growth measurements were normalized to plateau elongation rates in 50 ng/ml BDNF. (D) and (E) show quantitation of axon growth in response to laminin. Bcl-2-expressing RGCs were cultured on PDL-coated tissue culture wells with BDNF (50 ng/ml) and a laminin-adsorbed substrate, as indicated, in the (D) absence or (E) presence of forskolin (5 μM). *, statistically different from control condition; **, statistically different from control and from (*) conditions (Dunnett's, $p < 0.01$).

was enhanced to over 4-fold when cAMP levels were elevated with forskolin (Figure 3E). Laminin-2 (merosin) produced identical results, and other matrix molecules also produced qualitatively similar results (data not shown). These findings demonstrate that matrix molecules significantly promote peptide-mediated axon growth, but they are unable to induce axon growth on their own.

Ability of Different Cell Types along the Visual Pathway to Stimulate Axon Growth

We next investigated which cell types in the developing visual pathway are able to induce axon growth by RGCs. We focused on E18 RGCs, an age when RGCs are normally extending their axons along the visual pathway. We first asked whether RGCs themselves secreted signals that promoted their own growth, as it has previously been found that they secrete soluble signals that promote their own survival (Meyer-Franke et al., 1995). We found that purified, bcl-2-expressing E18 RGCs cultured at high density rather than clonal density robustly extended axons despite the complete absence of any added neurotrophic support (Figure 4A). RGCs were able to induce in each other small but significant axon growth at densities as low as 10 neurons/mm^2 (Student's

Table 1. RGC Axon Growth

Condition	Length (%) ± SEM
Control	4 ± 2
Growth media	144 ± 31
Peptides	
NGF	32 ± 7.2
BDNF	100 ± 11
NT-3	25 ± 12
NT-4	64 ± 6.8
IGF-1	57 ± 7.3
Insulin	52 ± 6.8
CNTF	107 ± 14
LIF	95 ± 26
FGF-1	28 ± 5.7
FGF-2	37 ± 5.1
BMP-2	12 ± 4.5
Pleiotrophin	8 ± 3.2
GGF/NRG-1	20 ± 12
GDNF	38 ± 10
Netrin-1	8 ± 4
Hormones	
Growth hormone	17 ± 7.3
Retinoic acid	12 ± 4
Substrates	
Laminin-1	12 ± 3
Merosin	15 ± 6
Collagen-III	6 ± 4
Collagen-IV	5 ± 4
Matrigel	11 ± 4
N-cadherin	14 ± 5

RGCs were purified, infected with adeno-bcl-2, and plated at clonal density (<5 RGCs/mm^2) in media as marked. All media contained forskolin (5 μM) to elevated cAMP, and RGCs were cultured on PDL and laminin-1 with the exception of control and substrates experiments, in which RGCs were cultured on PDL and various protein substrates if indicated. Growth Media contains BDNF, CNTF, and IGF-1. All growth factors were used at 10 ng/ml. The longest axon on each RGC was counted, and average axon lengths were normalized to the BDNF condition.

t test, $p < 0.05$). Consistent with this finding, medium conditioned by embryonic RGCs also stimulated axon growth (Figure 4B).

Medium conditioned by target cells, but not optic nerve or retinal cells, has previously been reported to strongly stimulate embryonic RGC survival (Johnson et al., 1986; Hofer and Barde, 1988; Meyer-Franke et al., 1995). To find out whether medium conditioned by retinal, optic nerve, or superior collicular target cells would also promote axon growth, we cultured purified E20 RGCs in serum-free medium conditioned by cells isolated from these three tissues (see Experimental Procedures) and measured axon length after 3 days. RGC axon growth was strongly stimulated by target cells, but remarkably we found that axon growth was equally well induced by retinal and optic nerve conditioned medium as well. To find out specifically which cell types secreted this axon growth activity, we next tested medium conditioned by purified optic nerve cell types. We found that optic nerve astrocytes and their precursor cells, as well as oligodendrocytes and their precursor cells, all strongly promoted axon growth (Figure 4B).

Because so many peptide factors and conditioned media were sufficient to promote axon outgrowth in RGCs, we hypothesized that one or a few signaling pathways might provide a common downstream mechanism for these various signals, as recently shown by Atwal et al. (2000) for peripheral neurons. We pharmacologically blocked two of these pathways, using PD98059 to inhibit ERK (extracellular regulated kinase) activity and LY to inhibit PI3K (phosphoinositide 3-kinase) signaling. RGCs cultured in Growth Medium show a significant MAPK (mitogen-activated protein kinase) dependence for survival (Meyer-Franke et al., 1995). Neither MAPK nor PI3K inhibition, however, had any effect on survival in bcl-2-expressing RGCs (Figure 4C). We then asked whether bcl-2-expressing RGCs stimulated with BDNF, CNTF, and IGF-1 depended on MAPK or PI3K signaling for axon growth, and found that inhibiting either pathway only partially decreased axon outgrowth in response to these peptides (Figure 4C). Blocking both kinase families simultaneously was sufficient to fully inhibit axon growth, however, suggesting that the two signaling molecules collaborate to bring about axon growth in response to a variety of peptide signals.

Unlike RGCs after E20, RGCs that are younger than E19 do not grow axons in response to BDNF or CNTF. From E15 to E18, RGCs are extending their axons through the optic nerve, and during this period, astrocyte precursor cells (APCs) are the predominant cell type in the optic nerve (Mi and Barres, 1999). As target cells in the superior colliculus are too distant to support RGC axon outgrowth at this age, we wondered whether APCs secrete signals that stimulate axon growth. When we purified APCs from E17 nerves and blocked signaling from BDNF, CNTF, and LIF using function-blocking antibodies, an unidentified factor or factors from APCs was able to strongly stimulate embryonic RGC axon growth as well as RGC survival (Figure 4D). These data suggest that APCs may normally promote survival and axon growth as RGCs extend toward their distal neuronal targets and point to the existence of important trophic stimuli that are not yet identified.

Role of Depolarization in Stimulating Axon Growth

We next investigated the role of electrical activity in stimulating axon growth. Physiological levels of electrical activity greatly enhance the ability of RGCs in retinal explants to respond to BDNF and other peptide trophic factors (Shen et al., 1999). To find out whether electrical activity regulates axon growth, we first investigated whether depolarization induced by high levels of extracellular K^+ (40 mM) would potentiate axon growth stimulated by BDNF and other trophic factors. We found that depolarization mimicked the ability of cAMP elevation to potentiate BDNF-stimulated axon growth (Figure 5A). This potentiation was abolished by the protein kinase A inhibitors Rp-cAMP and H89. Thus, prolonged depolarization potentiates BDNF-induced axon growth, and does so by elevating cAMP.

Both depolarization and cAMP elevation rapidly increase surface levels of the BDNF receptor TrkB on neuronal somas, axons, and growth cones within minutes (Meyer-Franke et al., 1998). If cAMP elevates BDNF responsiveness by increasing TrkB receptors at the surface, then forcing TrkB surface expression should be sufficient to enhance responsiveness in the absence of cAMP elevation. To test this hypothesis, we increased TrkB expression by using an adenoviral vector that pro-

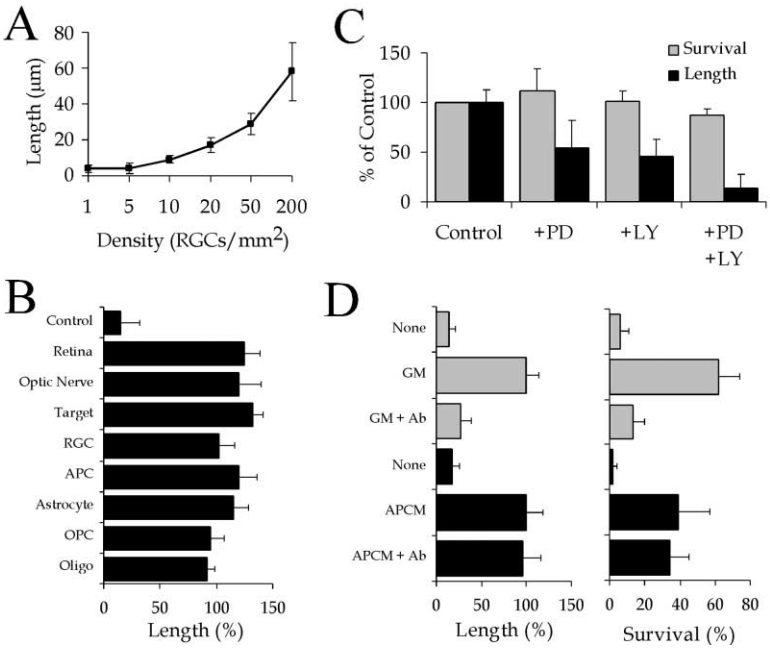

Figure 4. Ability of Visual Pathway Cells to Induce Axon Growth

(A) Purified RGCs infected with adeno-bcl-2 were cultured for 3 days at clonal (1 neuron/mm^2) and higher densities, and the longest axon was measured. (B) Axon growth of purified RGCs infected with adeno-bcl-2 and cultured at <5 neurons/mm^2 in the presence of conditioned media as indicated, and normalized to axon growth in Growth Media. APC, astrocyte precursor cells; OPC, oligodendrocytes precursor cells. (C) Bcl-2-expressing RGCs were cultured in Growth Media in the presence of PD98059 (50 μM) or LY294002 (50 μM) as marked, and their survival and axon growth were measured after 3 days. (D) E20 RGCs (gray bars) extended axons (left) and survived (right) in response to Growth Media. A cocktail of function-blocking BDNF, CNTF, and LIF antibodies, *Ab*, abolished this E20 growth response. E17 RGC axon growth (black bars, left) and survival (right) in response to media conditioned by astrocyte precursor cells (APCM) were not blocked by these antibodies.

motes the expression of *TrkB* fused to enhanced-GFP (Watson et al., 1999). Bcl-2-expressing RGCs overexpressing TrkB responded to BDNF by growing axons in the absence of depolarization or cAMP elevation (Figures 5B and 5C). A similar enhancement of survival responsiveness was also observed: in the presence of BDNF, TrkB increased survival from 4% ± 2% to 38% ± 5% (mean ± SEM). TrkB overexpression did not, however, increase axon growth significantly in the presence of cAMP elevation. These results show that elevation of surface levels of TrkB is sufficient to fully mimic the ability of cAMP elevation to enhance BDNF responsiveness.

Role of Physiological Levels of Activity in Stimulating Axon Growth

To investigate whether more physiologic levels of activity would affect axon outgrowth, we took three approaches. First, we asked whether intermittent depolarization by elevated K^+ over the course of several hours increased axon outgrowth in response to BDNF. We visualized RGCs using time lapse microscopy and applied short, 0.5 s pulses of 40 mM KCl to the bath once every 2 min to mimic the approximate time course of their excitation in the developing retina (Wong et al., 1993, 1998). We loaded RGCs with the calcium indicator dye fura-2 and found that RGCs remained responsive to intermittent depolarizing stimuli by fluxing calcium in response to KCl pulses over the course of hours (Figure 6A). When we measured axon growth in bath-applied trophic factors with and without intermittent KCl depolarization, we found that intermittent KCl stimulation dramatically potentiated peptide factor-induced axon growth (Figure 6B).

In our second approach, we examined the effect of stimulating RGCs in culture to fire action potentials in a pattern similar to their normal activity in embryonic retina. RGCs are normally electrically active before photoreception begins, during the period when they are extending their axons through the optic nerve to their targets. During this time period, RGCs within the retina exhibit intermittent bursts of action potentials separated by 1–2 min intervals of silence (Wong et al., 1998). Thus, we used direct electrical stimulation to test whether it would similarly enhance axon growth in response to peptide trophic factors. We cultured RGCs on silicon chips containing multiple electrodes through which we could apply field stimulation (Figure 7A; Borkholder et al., 1997). We used a stimulation protocol that closely mimicked the physiologic activity RGCs experience normally during the embryonic and early postnatal period, characterized by short bursts of action potentials separated by periods of relative silence (Figure 7A). RGCs were visualized by labeling with DiI (Figure 7B) and their survival was confirmed by labeling with calcein. Electrical stimulation on its own had no effect on RGC survival or axon growth in the absence of peptide trophic factors (data not shown). Remarkably, we found that even these very low levels of electrical stimulation profoundly potentiated RGC axon growth (Figures 7B and 7C) and survival (Figures 7D and 7E) in response to peptide factors. SQ22536, an adenylate cyclase inhibitor, or TTX (tetrodotoxin) largely blocked the ability of physiologic stimulation to enhance responsiveness to peptide trophic factors, although these only slightly affected baseline levels of BDNF-induced axon growth in the absence of stimulation (Figure 7C). We repeated these experiments using RGCs purified from embryonic day 19–20 rats, a developmental age at which RGCs are still normally extending their axons toward their targets in vivo. Embryonic RGCs cultured in the presence of BDNF showed a similar 6-fold enhancement of axon length in response to electrical stimulation, which was both action potential (TTX) and cAMP (SQ) dependent (data not shown). These results show that physiological levels of electrical activity promote peptide factor-induced out-

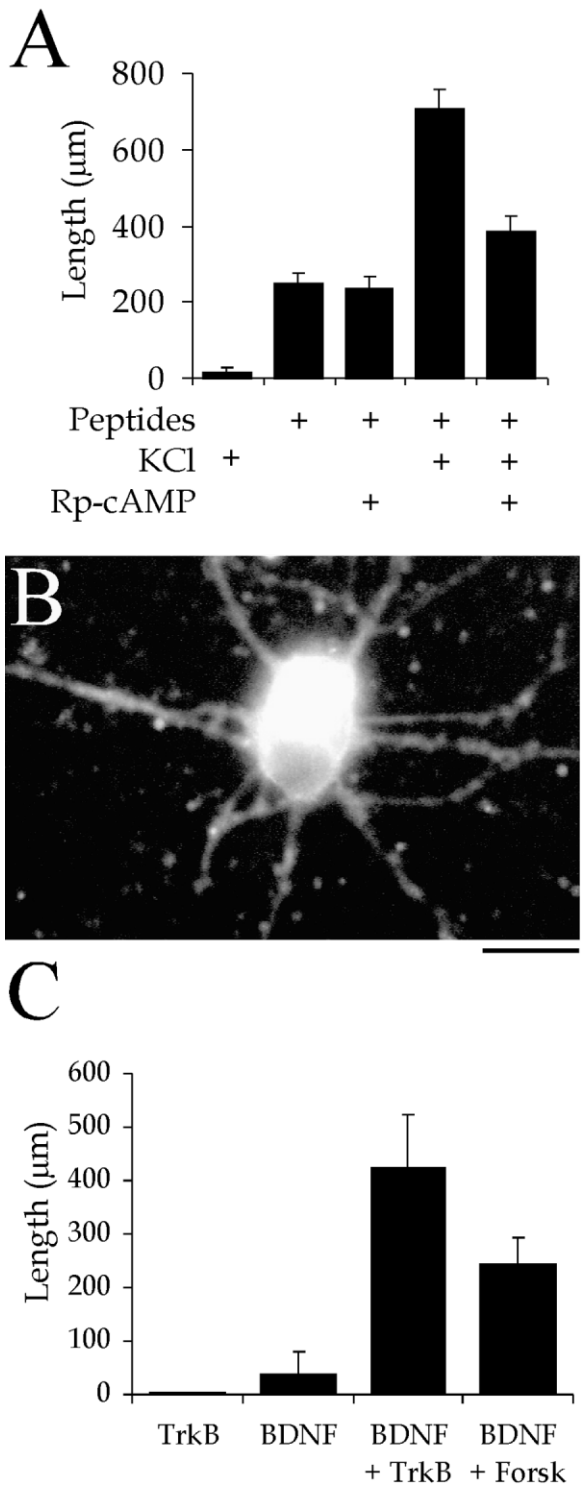

Figure 5. Effects of Depolarization-Induced cAMP Elevation on Axon Growth

(A) Purified RGCs were infected with adeno-bcl-2, cultured at clonal density (<5 neurons/mm^2) for 3 days, and then labeled with the fluorescent vital dye calcein-AM. Peptides: BDNF, CNTF, insulin; KCl (40 mM); Rp-cAMP (10 μM). (B) RGCs were infected with adeno-TrkB-eGFP and adeno-bcl-2 and cultured for 3 days; eGFP fluorescence shown here reveals high levels of TrkB throughout the cell body and processes. Scale bar 50 μm. (C) Bcl-2-expressing RGCs' mean longest axon on each RGC infected with adeno-TrkB-eGFP and/or cultured in BDNF as marked.

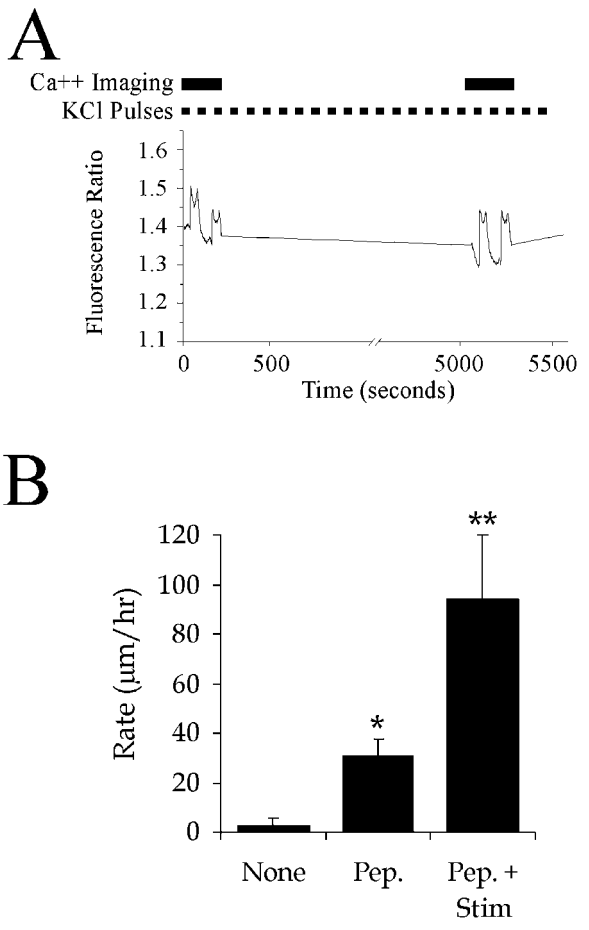

Figure 6. Effects of Intermittent Depolarization on Axon Growth

Purified RGCs were infected with adeno-bcl-2 and cultured for 1–2 days in growth media. After transfer to the time lapse stage, RGCs were recovered for 1–2 hr in a minimal media, switched into media containing BDNF, CNTF, and insulin, and visualized under time lapse brightfield microscopy in the presence or absence of KCl pulses. (A) RGCs loaded with the calcium-responsive fluorophore fura-2-AM were stimulated with intermittent pulses of KCl as described in the text and visualized at the beginning and end of the culture period. Upward deflections reflect influx of calcium in response to KCl (arbitrary units). (C) shows quantitation of net axon growth over time lapse periods ranging from 1–2 hr.

growth in a cAMP-dependent and in an action potential-dependent manner both during development as well as at older ages.

Finally, we tested whether endogenous retinal activity would increase axon outgrowth from retinal explants. Endogenous waves of activity sweep across developing mammalian retinas and depolarize RGCs (Wong et al., 1993; Feller et al., 1996), and these waves continue in explanted postnatal rat retinas in culture (J.L.G. and D. Stellwagen, unpublished observations). Postnatal retinas were explanted and cultured ganglion cell layer-side down on nitrocellulose filters, which aid retinal adhesion to the substrate. Axons growing from retinal explants have previously been demonstrated to originate from RGCs (Bahr et al., 1988). In the presence of BDNF, retinal axons exited the retinas and grew out onto the filters (Figure 8A). However, BDNF-induced axon

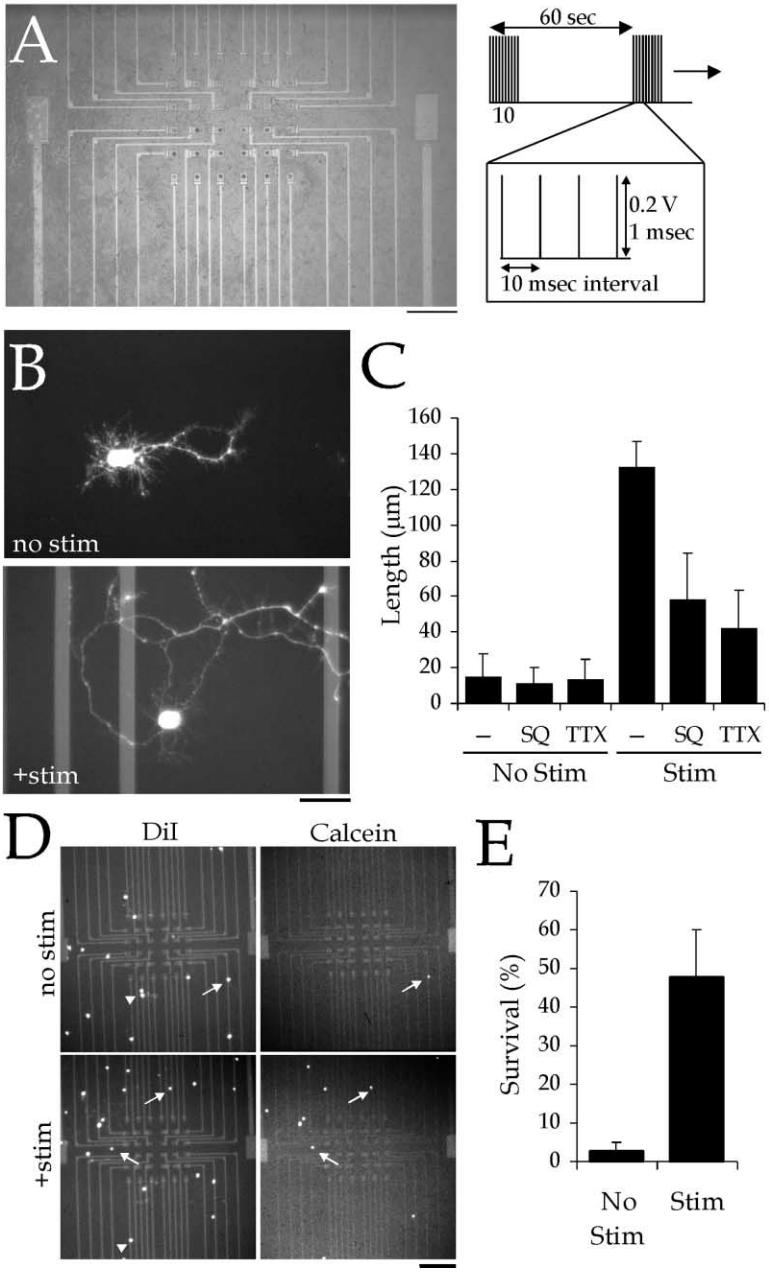

Figure 7. Effects of Electrical Activity on Axon Growth and Survival in Response to BDNF

(A) Photograph of silicon chip growth substrate, and diagram of burst-stimulation protocol (see Experimental Procedures). (B) DiI-labeled RGCs cultured on silicon chips extended rudimentary axons in response to BDNF (top). Axon growth was enhanced if the neurons were stimulated with electrical activity (bottom). (C) Mean longest axon/RGC ± SEM measured after 16 hr in the presence of BDNF, with stimulation, SQ, or TTX as marked and described in the text. (D) DiI-labeled RGCs (left panels) were plated in media containing BDNF and stimulated by electrical activity as marked. RGC survival was assessed 48 hr later by labeling with calcein-AM (right panels). DiI-labeled RGCs stained with calcein are alive (arrows); those lacking calcein are dead (arrowheads). (E) RGC survival in response to BDNF was enhanced 10-fold by electrical stimulation. Scale bar for (B) 50 μm, (D) 200 μm.

extension was reduced 3-fold in the presence of a cocktail of activity blockers including TTX, curare, and kynurenic acid (Figures 8B and 8C), or in the presence of the adenylate cyclase inhibitor SQ22536 or the PKA (protein kinase A) inhibitor Rp-cAMP (Figure 8C). In the presence of activity blockade or of adenylate cyclase inhibition, the nonhydrolyzable cAMP analog CPT-cAMP rescued axon extension from the explants, although CPT-cAMP could not induce any outgrowth on its own (Figure 8C). When we repeated these experiments with embryonic retinas, we obtained the same result (data not shown). These data demonstrate that endogenous levels of synaptic and electrical activity enhance retinal axon growth in response to BDNF.

Discussion

Retinal Ganglion Cells Do Not Extend Axons by Default

By elevating expression of the anti-apoptotic gene *bcl-2* in RGCs, we found that we could separate the ability of a neuron to survive from its ability to extend axons. Bcl-2 overexpression alone prevented RGCs from undergoing apoptosis when cultured in the complete absence of neurotrophic signals. These bcl-2-overexpressing RGCs failed to elaborate axons or dendrites despite maintenance of a normal soma size. These data demonstrate that surviving neurons do not extend axons by default and show that axon growth must be specifically sig-

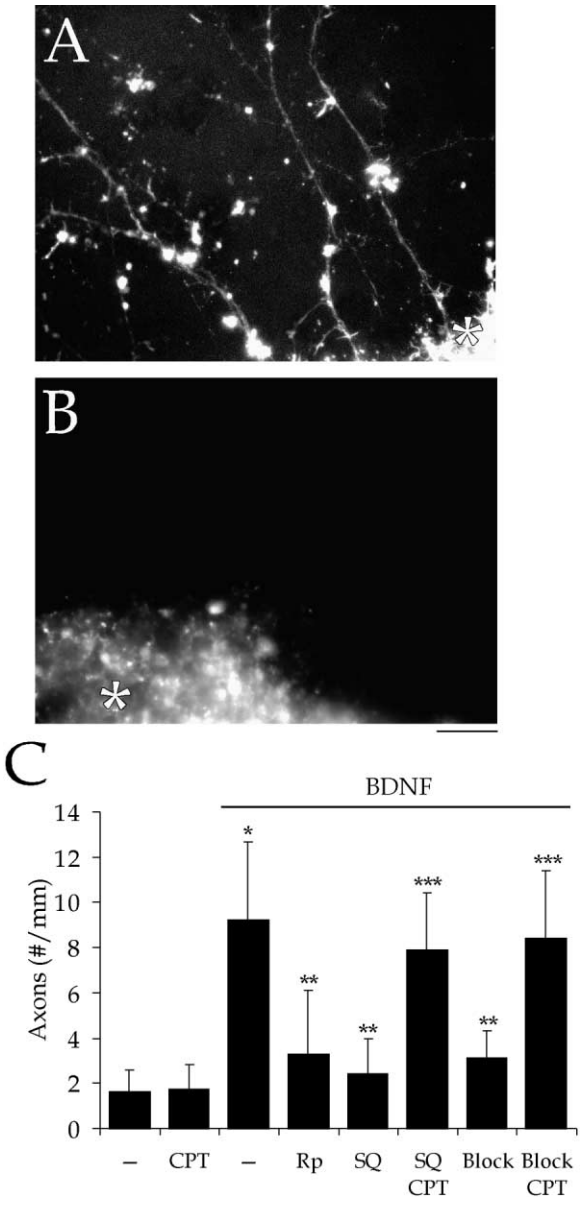

Figure 8. Effect of Endogenous Retinal Activity on Axon Growth

Retinas were cultured on nitrocellulose in the presence of BDNF as described in the text. After 3 days, retinas and axons were visualized by staining with fluorescent phallotoxins (Molecular Probes). (A) In the presence of BDNF, axons grew out from retinal explants (marked with asterisk). (B) The addition of a cocktail of activity blockers decreased axon outgrowth in response to BDNF. Scale bar 50 μm. (C) After 3 days, the number of axons extending from each retina was counted, and normalized to the border length of retina exposed. BDNF (50 ng/ml) was present in the media as marked. *, BDNF induced significant outgrowth; **, Rp, SQ or activity blockade (Block) eliminated this outgrowth; ***, CPT rescued the loss of axon growth seen with SQ or activity blockade to levels not different from control (Dunnett's, $p < 0.01$).

naled. These results concerning RGC axon growth are in good accord with previous findings on PNS axon growth (Deckwerth et al., 1996; Lindsay, 1988; Lindsay et al., 1985) in showing that neuronal survival alone is not sufficient to elicit robust axon growth.

These data also provide a model system in which the extracellular signals that normally promote axon growth can be elucidated, as well as the intracellular mechanisms by which they act. Importantly, the vast majority of embryonic and postnatal RGCs that we studied had already initiated dendritic and axonal growth in vivo before the time of isolation. Although the neuronal purification shears off axons and dendrites, typically RGCs retain a remnant of the axon and dendrite hillocks (our unpublished observations). Thus our studies focus specifically on the mechanisms that induce axonal elongation and leave open the question of how axon initiation is signaled.

Although most studies have implicated bcl-2 in promotion of cell survival, bcl-2 was recently reported to be an intrinsic genetic switch required for axon regeneration by RGCs (Chen et al., 1997). Our studies do not provide support for this function. Not only is overexpression of bcl-2 not sufficient to induce axon growth by embryonic or postnatal RGCs, but also, in a separate series of experiments in the presence of RGC growth media, we found it does not enhance axon growth, axon number, or the amount of branching. Similarly, axons appear to extend normally in mice with a targeted disruption of the *bcl-2* gene (Michaelidis et al., 1996) and bcl-2 overexpression does not promote regeneration of axotomized RGCs in vivo (Chierzi et al., 1999; Lodovichi et al., 2001). Thus, the ability of bcl-2 to enhance the numbers of regenerating RGCs after injury (Chen et al., 1997) is most likely accounted for by its ability to promote the survival of RGCs that would normally die when injured and disconnected from their target-derived trophic signals.

Peptide Neurotrophic Factors Are Necessary and Sufficient to Signal Axon Growth and Matrix Molecules Are Not

Laminin is the prototypical axon outgrowth-promoting molecule (Lander et al., 1985a, 1985b), and its growth-promoting and adhesive properties are biologically separable (Calof et al., 1994). Similarly L1 and N-cadherin have been found to strongly promote axon growth (Bixby et al., 1988; Lochter and Schachner, 1993). We were therefore surprised to find that these molecules were not sufficient to induce any axon growth on their own. Instead, we observed that laminin and other substrate molecules strongly potentiated axon growth rates in response to peptide trophic signals. Integrins may activate a number of intracellular signaling pathways (Schwartz and Baron, 1999), and potentiate mitogenic signaling by tyrosine kinase receptors (Hynes, 1992; Aplin et al., 1999, 2001). Although integrin signaling increases cAMP levels in growth cones (Gomez et al., 2001), we found that Rp-cAMP failed to block laminin's potentiation of BDNF-induced axon growth, and that elevation of cAMP synergized with laminin in potentiating BDNF-induced axon growth, suggesting that laminin and cAMP have separate actions. As integrin receptors have been shown to exhibit multiple independent binding and functional determinants (Neugebauer and Reichardt, 1991), we hypothesize that laminin's effect is specific to the cytoskeletal dynamics of growth cone procession (Suter and Forscher, 2000) and the modulation of axon guid-

ance (Hopker et al., 1999), whereas the effect of elevating cAMP is to globally increase neurotrophin responsiveness and thereby to activate a generalized program of axon growth at the growth cone, along the axon, and at the cell soma.

In contrast to matrix molecules, we found that single neurotrophins and other peptide trophic factors were sufficient to induce robust axon growth on their own. Recent studies have suggested that CNTF is better able to induce axon growth and regeneration than BDNF is in vitro and in vivo (Cui et al., 1999; Jo et al., 1999). Our studies show that BDNF and CNTF have the same ability to directly signal axon outgrowth by RGCs. The differences between our findings and previous reports could be accounted for by a differential ability of CNTF and BDNF to signal glial cells to promote or inhibit axon growth and indirectly mediate trophic effects in vivo (Harada et al., 2000).

Given that the signaling mechanisms that promote cell survival and axon growth differ, as for instance shown by the ability of bcl-2 to promote cell survival but not axon outgrowth, it is remarkable that the same peptides that most strongly promote cell survival are generally the same ones that most strongly promote axon growth. The survival and axon growth responses to a single peptide, BDNF, displayed similar dosage sensitivities, suggesting that the threshold for survival signaling is similar to that of axon growth signaling. Apparently, the mechanisms that promote survival and axon outgrowth share the same upstream elements such as receptor activation but diverge downstream. For example, we found that in response to BDNF, MEK but not PI3K is required for survival (Meyer-Franke et al., 1998), whereas both helped to mediate axon outgrowth. Bcl-2-expressing RGCs should provide a good model system to investigate the identity of these downstream mechanisms.

All Optic Nerve Glial Cell Types Have the Ability to Strongly Promote Axon Growth

In the present study, we show that astrocytes and oligodendrocytes, as well as astrocyte precursor cells and oligodendrocyte precursor cells, secrete signals that strongly promote RGC axon outgrowth. In addition, we found that APCs secrete a strong survival-promoting activity that is not neutralized by anti-BDNF, CNTF, or LIF antisera. Thus, just as the floor plate has been shown to provide survival support to developing spinal commissural neurons passing by (Wang and Tessier-Lavigne, 1999), we hypothesize that optic nerve APCs provide analogous en passant trophic signals that promote the survival of embryonic RGCs and the growth of their axons early in development as they grow toward their targets. Retinal signals are also sufficient to induce embryonic RGC survival (J.L.G., unpublished observations), suggesting that the primary function of glial-derived trophic support is to enhance the growth and survival of axons as they extend along their pathway. In addition, glial-stimulated axon growth may be crucial in achieving axonal regeneration after injury (Goldberg and Barres, 2000). Because we found that RGCs themselves secrete a strong axon growth-promoting activity, en passant signaling of survival and axon growth may also be mediated by the release of trophic factors from their axons as they travel together along the visual pathway during development. As E17 RGCs do not respond to any of the so far identified trophic peptides, yet their growth is strongly stimulated by APCs and target cells, it is likely that important axon growth-stimulating signals for RGCs remain to be identified.

Physiological Levels of Activity Profoundly Enhance Axon Growth by Enhancing Neurotrophic Responsiveness of RGCs

We found that electrical activity dramatically enhanced the rate of trophic factor-stimulated axon growth by RGCs both in vitro and within retinal explants. Little axonal growth was elicited by peptide neurotrophic factors unless the cells were electrically active or their intracellular levels of cAMP were elevated pharmacologically. Most striking was that, for RGCs cultured directly on silicon chips through which we could control the level and pattern of activity, exceedingly low levels of electrical stimulation, comprised of short bursts once every minute, were sufficient to dramatically potentiate axon growth and survival in response to peptide neurotrophic factors, although activity on its own had no effect. In this respect, RGCs are unlike PNS neurons, which survive and extend axons well in response to neurotrophins without electrical stimulation (Levi-Montalcini, 1987; Barde, 1989, 1990). The response to activity may differ generally between CNS and PNS neurons (Goldberg and Barres, 2000).

These observations raise the question of whether electrical activity normally enhances the rate of axon growth by developing CNS neurons in vivo. During development, electrical activity appears not to be essential for at least some axon growth (Harris, 1980; Verhage et al., 2000). In addition, a number of studies suggest that activity may decrease the rate of axon growth, for instance by increasing the rate of axon arborization by stabilizing growing branches (Rashid and Cambray-Deakin, 1992; Cohen-Cory, 1999; Cantallops et al., 2000), or by inhibiting axon growth transiently at choice points (Gomez and Spitzer, 1999). In fact, growth cones may collapse acutely but then desensitize to electrical stimulation and recommence axon growth (Fields et al., 1990). Taken together with our results that electrical activity greatly enhances the rate of trophic factor-stimulated axon growth in vitro and in retinal explants, we hypothesize that after a brief accommodation at the growth cone, the longer-term effect of electrical stimulation is to greatly promote the rate of axon growth. Such a mechanism may give active neurons a growth advantage over less active neurons during development, and changes in neuronal activity could contribute to the failure of trophic signaling after axotomy (Shen et al., 1999).

How does electrical activity enhance responsiveness to BDNF and other trophic factors in RGCs? In RGCs and in hippocampal neurons, electrical activity rapidly upregulates surface levels of TrkB by translocation of receptors from an intracellular store and increases BDNF responsiveness (Meyer-Franke et al., 1998; Du et al., 2000). Here, the ability of electrical activity to promote neurotrophin responsiveness appears to be accounted for by elevation of cAMP, as adenylyl cyclase inhibition blocks the activity-dependent potentiation.

Consistent with this possibility, we found that overexpression of TrkB was sufficient to mimic the effects of cAMP elevation and activity in promoting BDNF responsiveness. However, as cAMP elevation also potentiates RGC axon growth in response to many other peptide trophic factors, cAMP elevation probably activates a generalized mechanism for increasing trophic responsiveness. We do not know whether this involves a similar upregulated secretion of a broad distribution of trophic receptors to the surface.

Is Electrical Activity Necessary for Axonal Regeneration?

An important implication of our findings is that the growth of axons of CNS neurons in vivo may also depend on local regulation by cAMP levels and electrical activity. Active neurons would thus survive and extend axons in response to peptide trophic stimulation better than silent or less active neurons. The fact that neurons need to be specifically signaled to reextend their axon, combined with the observation that such signals may have to be applied locally at the growth cone (Campenot, 1994), may explain why some CNS neurons survive in an atrophied state after axotomy but fail to regenerate their axons. For example, Purkinje cells resist death but fail to regenerate after axotomy (Dusart and Sotelo, 1994), as do many of the corticospinal motor neurons that project down the spinal cord (Schwab and Bartholdi, 1996). Such neurons may receive sufficient signals to mediate survival of the cell body, presumably from collateral axon branches that could still relay target-derived trophic stimuli (Bernstein-Goral and Bregman, 1997), but insufficient signals to locally induce axon elongation at the severed end. If axon growth normally depends on activity in vivo, and if damaged cells are less active, a CNS neuron's ability to regenerate its axon could be impaired, even in the presence of exogenously applied trophic support (Shen et al., 1999). Previous studies have suggested that trophic factor delivery alone or electrical stimulation alone do not induce axonal regeneration. Our findings suggest that to elicit successful CNS regeneration, it may be crucial to simultaneously provide trophic peptides as well as signals such as cAMP or activity to ensure optimal responsiveness.

Experimental Procedures

Detailed step-by-step protocols for all procedures are available upon request (barres@stanford.edu).

Reagents

Regeneron generously provided several recombinant trophic factors (human BDNF, CNTF). Other recombinant peptide trophic factors were obtained from Peprotech (Princeton, NJ). S. Brady-Kalley generously provided purified N-cadherin (Burden-Gulley and Brady-Kalnay, 1999); and M. Lin and M. Greenberg generously provided the adenoviral TrkB-GFP vector.

Purification and Culture of RGCs

RGCs from P8 Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) were purified by sequential immunopanning to 99.5% purity (Barres et al., 1988) and cultured on poly-D-lysine (PDL; 70 kDa, 10 μg/ml; Sigma) and laminin (2 mg/ml; Telios/Gibco) or other substrates as noted; N-cadherin was also tested adsorbed to nitrocellulose as described (Burden-Gulley and Brady-Kalnay, 1999). RGCs were cultured in serum-free defined medium as described (Meyer-Franke et al., 1995). RGC Growth Medium contained BDNF (50 ng/ml), CNTF (10 ng/ml), insulin (5 μg/ml), and forskolin (5 μM).

Survival and Immunofluorescence Assays

MTT survival assays (Mosmann, 1983) were performed as described (Meyer-Franke et al., 1995). All values are given as mean ± SEM of at least three cultures. RGCs were immunostained by incubating paraformaldehyde-fixed cultures overnight at 4°C in anti-bcl2 antiserum (human-specific, PharMingen) and then for 1 hr at 25°C in Alexa-conjugated secondary antibodies (Molecular Probes), using typical protocols (Meyer-Franke et al., 1995).

Quantitation of Axon Growth

RGCs were plated at about 500 cells/well in 24-well plates (about 3 cells/mm^2) in 500 μl of serum-free media in the presences of trophic or other factors as described in the text. After three days, the fluorogenic substrate calcein-AM (Molecular Probes) was added to a final concentration of 1 mg/ml. After 1 hr at 37°C, fluorescent, viable cells were visualized in a Nikon fluorescence microscope, and images were gathered with a Princeton Instruments CCD camera and collected and analyzed with MetaMorph (Universal Imaging). We measured axon lengths using an ocular grid and confirmed a subset of our measurements by counting pixels on computer-acquired images. In initial experiments, we found that blinded measurements by ocular grid differed by less than 5% from those measured by computer analysis. All data on axon lengths subsequently presented were measured by ocular grid, and standard errors do not reflect this 5% systematic error.

Preparation of Collicular, Retinal, Optic Nerve, and Purified Glial Cultures

Target cell suspensions were prepared by dissecting the superficial layer of the superior colliliculus, which includes the retinorecipient layer, digesting lightly in papain, and triturating into a single-cell suspension. We found that, when purified from P8 rats, this suspension was comprised of 15% neurons both acutely and after 3 days in culture, as assessed by MAP2 immunoreactivity (J.L.G. and Y. Hua, unpublished data). Suspensions of mixed retinal and mixed optic nerve cells were prepared in similar fashion. Optic nerve oligodendrocytes, astrocytes, oligodendrocyte precursors, and astrocyte precursors were purified by immunopanning as previously described (Barres and Raff, 1993; Barres et al., 1993; Mi and Barres, 1999). To test the effects of mixed retinal, glial, or target cells, or purified glial cell populations on axon growth, acutely prepared cells were cultured on porous inserts suspended directly above the RGCs. In some experiments, media were conditioned for 2–3 days in serum-free defined medium containing B27; conditioned medium was then added to cultures of acutely purified RGCs.

Time Lapse Imaging of Axon Growth

E20 RGCs were purified and infected with adeno-*bcl-2*, plated on PDL- and laminin-coated glass coverslips, recovered for 1 or 2 days in RGC Growth Medium, and then transferred to the stage of the CCD-equipped Nikon microscope as above. For calcium imaging, cultures were incubated at room temperature for 30 min in 1 μM fura-2-AM (Molecular Probes). Transferred coverslips were allowed to recover for 1 hr in minimal media without peptides before imaging. Time lapse cultures were maintained at 37°C and continuously perfused with CO_2-equilibrated culture media containing peptides BDNF, CNTF, and insulin. Pulses of KCl (40 mM, 500 ms) were applied to stimulate RGCs intermittently.

Electrical Stimulation on Silicon Chips

RGCs were labeled with DiI, infected with adeno-*bcl-2* as marked, and cultured on PDL- and laminin-coated silicon chips (generously provided by G. Kovacs; Borkholder et al., 1997). For stimulations, ten pulses (100 Hz, 0.2 mV) were applied once every 2 min using a digital stimulator (Cygnus Technologies).

Retinal Axon Outgrowth

P8 retinas were dissected, quartered, and adhered ganglion cell layer-side down onto nitrocellulose paper. Retinal fragments were cultured in the presence of BDNF and other pharmacologic agents

as marked. The number of axons extending from the border of the retinae was counted after 3 days in culture.

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