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Characterization of the Signaling Interactions That Promote the Survival and Growth of Developing Retinal Ganglion Cells in Culture

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Summary

The signaling mechanisms that control the survival of CNS neurons are poorly understood. Here we show that, in contrast to PNS neurons, the survival of purified postnatal rat retinal ganglion cells (RGCs) in vitro is not promoted by peptide trophic factors unless their intracellular cAMP is increased pharmacologically or they are depolarized by K^+ or glutamate agonists. Long-term survival of most RGCs in culture can be promoted by a combination of trophic factors normally produced along the visual pathway, including BDNF, CNTF, IGF1, an oligodendrocyte-derived protein, and forskolin. These results suggest that neurotransmitter stimulation and electrical activity enhance the survival of developing RGCs and raise the question of whether the survival control mechanisms of PNS and CNS neurons are different.

Introduction

Neurons, like most animal cells, require continuous stimulation by extracellular signals in order to survive (Raff, 1992; Raff et al., 1993). Such signals suppress a highly conserved, cytoplasmic cell suicide program (Raff et al., 1993). A competition for limited amounts of target-derived survival signals is thought to underlie the death of many CNS and PNS neurons during development (Levi-Montalcini, 1987; Purves, 1988; Barde, 1989), and recent evidence suggests that neurons continue to require survival signals in the adult (Acheson et al., 1995). The survival and growth of neuronal processes are regulated by similar signaling mechanisms (Campenot, 1994); control of survival and growth of axonal terminals is thought to underlie the activity-dependent formation of synaptic connections (Cowan et al., 1984; Cohen-Cory and Fraser, 1994; Cabelli et al., 1995). Thus, survival signaling mechanisms may help to control not only the number of neurons, but probably also their location, size, and connectivity (Purves, 1988; Raff et al., 1993). Understanding how the survival of CNS neurons and their processes is controlled is fundamental to understanding how the brain develops and repairs itself.

The mechanisms that control the survival of PNS neurons have received extensive study. Dorsal root ganglion sensory neurons, sympathetic neurons, and ciliary motor neurons can be purified and cultured. Their survival can be promoted by peptide trophic factors including ciliary neurotrophic factor (CNTF) and target-derived neurotrophins such as nerve growth factor (NGF; Levi-Montalcini, 1987; Barde, 1989, 1990). Compared with the PNS, however, less is known about the mechanisms that control the survival of most types of CNS neurons. Spinal motor neurons, a class of cholinergic CNS neuron that projects into the PNS, can be highly purified and cultured, and their survival is also promoted by target-derived neurotrophins and CNTF (Henderson et al., 1993; Hughes et al., 1993). Little is known about the mechanisms that control the survival of most CNS neurons, however, because it has been difficult to highly purify them. The survival requirements of most CNS neurons are so stringent and poorly understood that in no case has a defined type of glutamatergic or GABAergic CNS neuron been highly purified and cultured in serum-free medium for more than 24 hr in the absence of glial or other cell types (Baptista et al., 1994; Craig and Banker, 1994).

We have focused on rat retinal ganglion cells (RGCs), the glutamatergic projection neurons that relay visual signals from the retina to the rest of the brain. As is true of PNS neurons and spinal motor neurons, the survival of RGCs depends on target-derived signals. Nearly all RGCs in the rat project to the optic tectum (Linden and Perry, 1983). They are generated between embryonic day 14 (E14) and E20 (Bunt et al., 1983; Reese and Colello, 1992), and half of these die as they innervate the tectum between postnatal day 0 (P0) and P4 (Lam et al., 1982; Potts et al., 1982). The majority of RGCs die after optic nerve transection (Perry and Cowey, 1979; Linden and Perry, 1983), and in culture their survival is enhanced by tectal cells (McCaffery et al., 1982; Armson and Bennett, 1983). The tectum produces a neurotrophin, brain-derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989; Hofer et al., 1990; Hohn et al., 1990; Cohen-Cory and Fraser, 1994; Herzog et al., 1994), which promotes the survival of enriched embryonic RGCs (Johnson et al., 1986; Rodriguez-Tebar et al., 1989). Thus, developing RGCs may compete for limiting amounts of target-derived BDNF much as sympathetic neurons compete for NGF (Barde, 1989; Rodriguez-Tebar et al., 1989). Although BDNF is likely to play an important role in promoting the survival of developing RGCs, BDNF is insufficient to promote their survival in culture for more than several days (Johnson et al., 1986).

We show here that the survival of highly purified embryonic and postnatal RGCs in serum-free culture is not promoted by peptide trophic factors unless they are simultaneously treated with forskolin or membrane-permeable cAMP analogs to increase their intracellular cAMP, or are depolarized by K^+ or glutamate receptor agonists; both effects are blocked by protein kinase A inhibition. Whereas the survival of the embryonic RGCs appears to depend primarily on tectum-derived signals, the long-term survival of postnatal RGCs can be promoted by a collaboration of trophic factors previously shown to be produced by glial

Table 1. Survival of Purified Retinal Ganglion Cells in Culture

Factor	% Surviving Cells at 3 Days	
	No Forskolin	Forskolin
Nothing	0.8 ± 0.3	5.5 ± 1.0
Insulin	1.3 ± 0.4	16.2 ± 1.5
IGF1	1.5 ± 0.5	18.3 ± 1.2
BDNF	2.4 ± 0.5	35.8 ± 2.6
NT-4/5	2.1 ± 0.4	32.9 ± 2.8
CNTF	1.9 ± 0.6	29.7 ± 2.5
LIF	1.5 ± 0.4	28.1 ± 1.7
bFGF	2.0 ± 0.4	17.2 ± 1.9
TGFα	1.2 ± 0.5	13.3 ± 2.1

Approximately 5,000 purified P8 RGCs were plated in triplicate in merosin-coated 96-well Falcon plates in 100 μl of B-S medium without insulin, containing a plateau concentration (50 ng/ml; except for insulin, which was 5 μg/ml) of the appropriate factor. After 3 days of culture, the percentage of cells surviving in each well was determined using the MTT assay (mean ± SEM of three cultures; see Experimental Procedures).

and neuronal cells along the visual pathway: BDNF, CNTF, insulin-like growth factor 1 (IGF1), and a protein secreted by oligodendrocytes. RGCs appear to differ from PNS neurons in the requirement for elevated cAMP to be responsive to their survival factors.

Results

Effects of Peptide Trophic Factors on Retinal Ganglion Cell Survival In Vitro

To determine the survival effects of different peptide trophic factors on RGCs, we purified P8 RGCs to >99.5% purity by sequential immunopanning (Barres et al., 1988) and cultured the purified cells in serum-free medium in merosin-coated 96-well tissue culture plates (see Experimental Procedures). After 3 days of culture, we assessed their survival with the MTT assay (Mosmann, 1983; Barres et al., 1992, 1993a) (see Experimental Procedures). In the absence of added growth factors, even when cultured at high cell density, nearly all of the cells died within 3 days with the characteristic morphology of apoptosis (Wyllie et al., 1984) (Table 1). Both by phase-contrast microscopy and by fluorescence microscopy after propidium iodide staining, the cell cytoplasm and nucleus were usually seen to be shrunken, and the chromatin was highly condensed and sometimes fragmented.

Over the same 3 day culture period, high concentrations of single peptide growth factors previously shown to be produced along visual pathways and to promote survival of other neurons, including insulin, IGF1, BDNF, neurotrophin-3 (NT-3), NT-4/5, CNTF, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and transforming growth factor α (TGFα), did not promote the survival of more than 2% of the cells (Table 1). Elevation of intracellular cAMP level alone, either by addition of forskolin, an activator of adenylate cylase (Table 1), or by the membrane-permeable cAMP analog, chlorophenylthio-cAMP (CPT-cAMP; 100 μM), had little survival-promoting effect. The ability of individual peptide trophic factors to promote RGC survival, however, was amplified by forskolin (or equivalently by CPT-cAMP) by about 10-fold (Table 1). These factors included IGF1 and insulin at concentrations sufficiently high to activate IGF1 receptors (5 μg/ml; Sara and Hall, 1990), as well as BDNF, NT-4/5, CNTF, LIF, bFGF, and TGFα. Trophic factors that did not promote the survival of RGCs at a high concentration (50 ng/ml) in the presence of forskolin included NT-3, NGF, TGFβ1, TGFβ2, TGFβ3, glial cell–derived neurotrophic factor (GDNF), glial growth factor (GGF), interleukin-3 (IL-3), and IL-7. The effects of forskolin were not mimicked by the membrane-permeable cGMP analog 8-bromo-cGMP (1 mM), the nitric oxide donors S-nitroso-N-acetylpenicillamine (SNAP; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM), or phorbol 12-myristate 13-acetate (TPA; 10 nM).

To determine the survival-promoting effects of different concentrations of BDNF and CNTF on purified RGCs, we performed dose–response curves in the presence of forskolin (Figure 1A). The BDNF concentration that promoted half-maximal survival was about 2 ng/ml and for CNTF was about 200 pg/ml, similar to the values reported for rodent peripheral neurons.

Effects of Multiple Peptide Trophic Factors on Short- and Long-Term Retinal Ganglion Cell Survival In Vitro

Because single factors did not promote 100% survival of RGCs, even in the presence of elevated cAMP (see Table 1), we investigated whether combinations of the factors would improve short-term survival (in the presence of forskolin). The factors fell into at least three classes: insulin and IGF1; BDNF and NT-4/5; or CNTF and LIF. Combinations of plateau concentrations of both factors in a class did not give a significantly better survival than the individual factors alone (data not shown). In contrast, a combination of plateau concentrations of factors from different classes produced additive effects on survival (Figure 1B). Insulin plus BDNF, for example, was better than insulin or BDNF alone; insulin plus BDNF and CNTF was better than any two of these alone (Figure 1B). The majority of the cells could be saved, at least for 3 days, by the combination of forskolin, insulin, BDNF, and CNTF (Figure 1B). The addition of bFGF and TGFα did not promote a further increase in survival. Additive effects were observed when these factors were combined with CNTF (data not shown); however, these combinations were not nearly as effective as BDNF together with CNTF. The omission of merosin (or laminin, whose effects were indistinguishable) as the substrate, which has previously been reported to promote neurite outgrowth by postnatal RGCs (Cohen and Johnson, 1991), even if replaced with poly-D-lysine, resulted in significantly fewer surviving cells (Figure 1B). In the absence of forskolin (or CPT-cAMP), combinations of peptide factors did not promote the survival of most cells. For instance, survival in BDNF and insulin was about 10% but was increased to 55% when forskolin was present. Strikingly, in all cases, the rate and extent of process outgrowth correlated closely with the degree of survival.

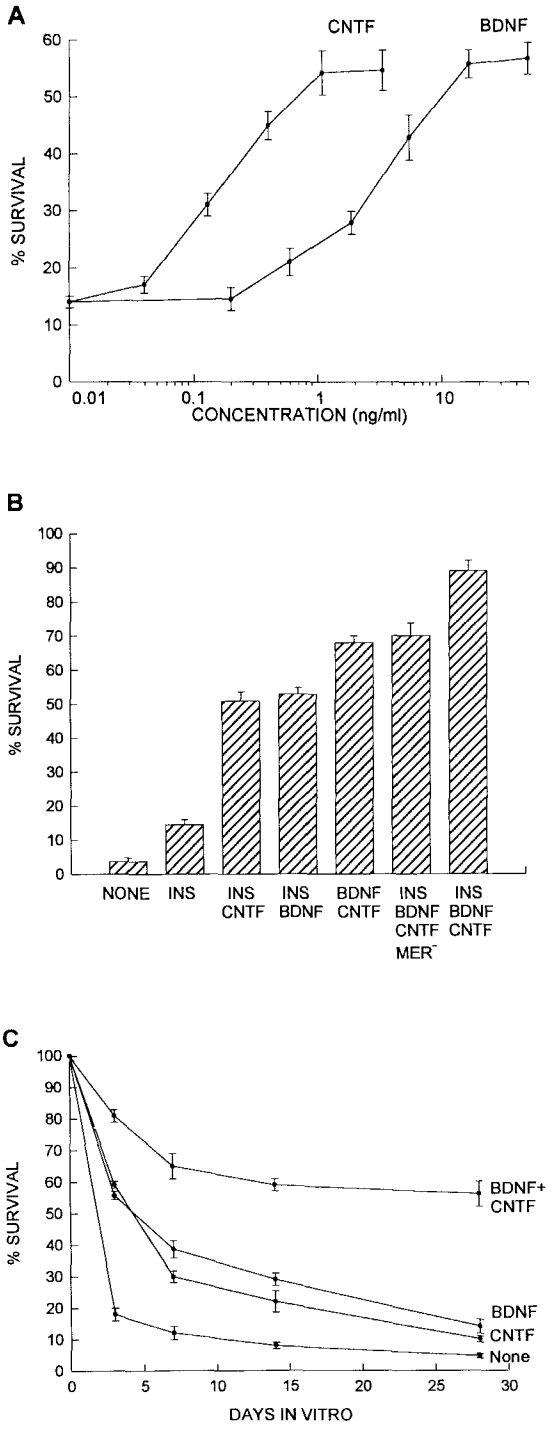

Figure 1. The Effects of Peptide Trophic Factors on Short-Term and Long-Term Retinal Ganglion Cell Survival

Purified retinal ganglion cells (RGCs) were cultured in serum-free B-S medium containing forskolin (5 μM) and factors as indicated for 3 days, unless otherwise indicated. The percentage of cells surviving was determined by the MTT assay (mean ± SEM of three cultures).

(A) Dose–response curves for BDNF and CNTF. The medium also contained insulin (5 μg/ml).

(B) The effects of combining survival factors on short-term RGC survival. All factors are used at plateau concentrations. Survival significantly increased as the total number of factors increased ($p < .02$); values with the same number of factors are not significantly different. Omission of merosin significantly decreased survival ($p < .02$).

(C) The effects of combining survival factors on long-term survival. The survival of purified RGCs cultured in the indicated factors was assessed after 3, 7, 14, and 28 days of culture. The medium also contained insulin (5 μg/ml).

We next investigated whether long-term survival of RGCs could be promoted by combinations of peptide trophic factors, in the presence of forskolin. As the number of factors that were combined was increased, we observed an increase in the percentage of cells that survived over short culture periods as well as over long-term periods (Figures 1C and 2). By 2 weeks in culture, the majority of cells had died when cultured in only one or two factors (e.g., BDNF and insulin). When all three factors were combined together (BDNF, CNTF, and insulin), the cells that survived for 1 week (about 63% of the cells) were able to survive for at least 1 month, the longest culture period that we studied (see Figure 1C). In contrast, after 1 month of culture, most cells cultured either in BDNF and insulin or in CNTF and insulin had died, strongly suggesting that BDNF and CNTF do not promote the survival of different subsets of cells, but act collaboratively on individual cells to promote long-term survival.

Effects of Cell Density on Retinal Ganglion Cell Survival In Vitro

We studied the effects of cell density on P8 RGC survival in medium containing BDNF, CNTF, high insulin, and forskolin. After 3 days in culture, survival at low density was nearly 3-fold lower than at high density (Figure 3A). To determine whether this effect was soluble or contact mediated, we cultured RGCs at low density on a glass coverslip positioned above a conditioning layer of high density RGCs (see Experimental Protocols). The survival of the low density cells with a conditioning layer was nearly identical to the survival of RGCs plated at high density (Figure 3A), suggesting that RGCs secrete soluble signals that promote their own survival. RGCs in vivo express *FGF* mRNA and protein (Elde et al., 1991; Connolly et al., 1992). To determine whether FGF could be the soluble autocrine activity, we studied its effects on cells cultured at low density. The survival of cells at low density was significantly increased by bFGF (Figure 3B), but not TGFα (data not shown), whereas the survival at high density was not significantly increased (Figure 3B). Furthermore, at clonal density, the addition of bFGF increased the survival of the cells by more than 5-fold. The survival of the cells at high density, however, was not significantly decreased by neutralizing anti-bFGF antibodies (data not shown), suggesting that bFGF itself is not the autocrine factor.

Intrinsic Properties of Purified Retinal Ganglion Cells in Culture

To determine how the properties of the purified RGCs in vitro compared to RGCs in vivo, we examined their morphology and electrophysiological properties. Most cells cultured for 1 week or more at high density had dendrites

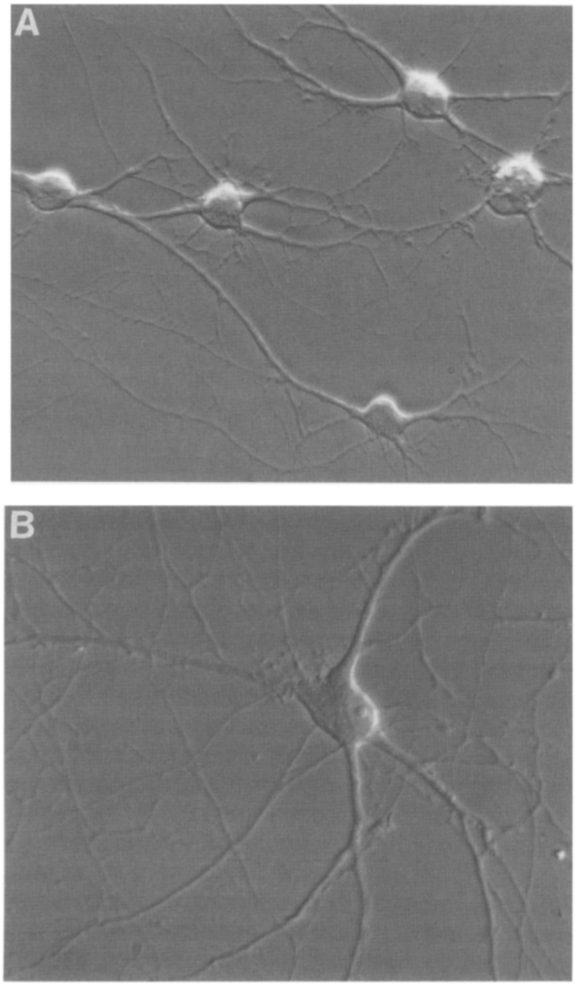

Figure 2. Morphology of Retinal Ganglion Cells in Culture

Photomicrographs of purified RGCs cultured in serum-free B-S medium containing BDNF, CNTF, IGF1, and forskolin for 1 week and visualized using Hoffman modulation contrast optics. Bar, 40 μm (A), 20 μm (B).

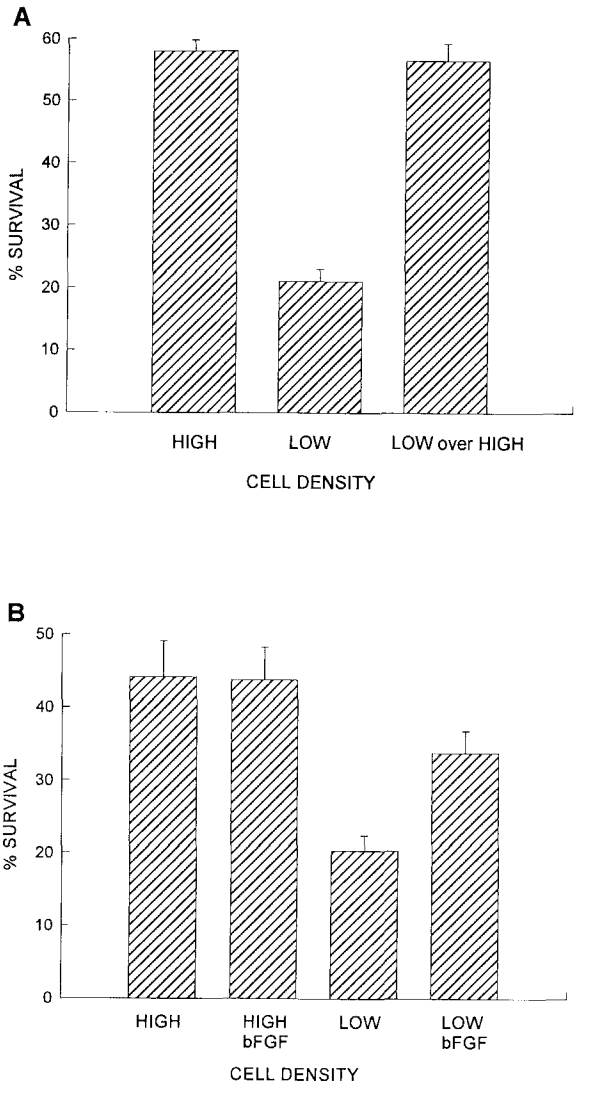

Figure 3. The Effect of Cell Density on Retinal Ganglion Cell Survival

Purified RGCs were cultured in serum-free B-S medium containing forskolin and plateau concentrations of BDNF, CNTF, insulin, and in some cases bFGF, as indicated for 3 (A) or 7 (B) days. The percentage of cells surviving was determined by the MTT assay. In (A), the cells are cultured either at high density (50,000 cells/well), at low density (1500 cells/well of a 24-well plate), or at low density above a conditioning layer of high density cells (see Experimental Procedures). In (B), cells are cultured at high and low density both in the presence and absence of bFGF. The presence of bFGF significantly enhanced survival ($p < .02$).

and axons (see Figure 2), as shown by immunostaining with the anti-MAP2 and anti-tau antibodies, respectively (Figures 4a and 4b) (Kosik and Finch, 1987). The number of dendrites per cell was generally about two to four, and these tended to be short, about 100 μm, and exhibited little or no branching. The number of axons per cell was typically one, but in some cases two or three axons could be observed.

To determine how the membrane properties of RGCs compare to those in vivo, we recorded from purified P8 RGCs that had been cultured for at least 1 week in serum-free medium containing BDNF, CNTF, insulin, and forskolin, using whole-cell patch-clamp recording. Their resting membrane potential was −57 ± 2 mV (mean ± SEM; n = 9). All of the cells fired repetitive action potentials in response to depolarizing current injection (n = 18; Figure 4C). Interestingly, the degree of spike adaptation among cells was highly variable, as previously reported for acutely isolated rat P8 RGCs, with some cells firing sustained trains of action potentials and others adapting quickly (Barres et al., 1988). Voltage-clamp recordings from 30 neurons in three different cultures revealed the occasional presence of spontaneous excitatory postsynaptic currents (Figure 4D). Their frequency was enhanced by the addition of K^+ (10 mM) to the bath solution; they were not eliminated by tetrodotoxin (TTX; 10 μM) but were eliminated by the glutamate antagonist kynurenic acid (1 mM; n = 10). Thus, the purified RGCs form at least some functional glutamatergic synapses in culture.

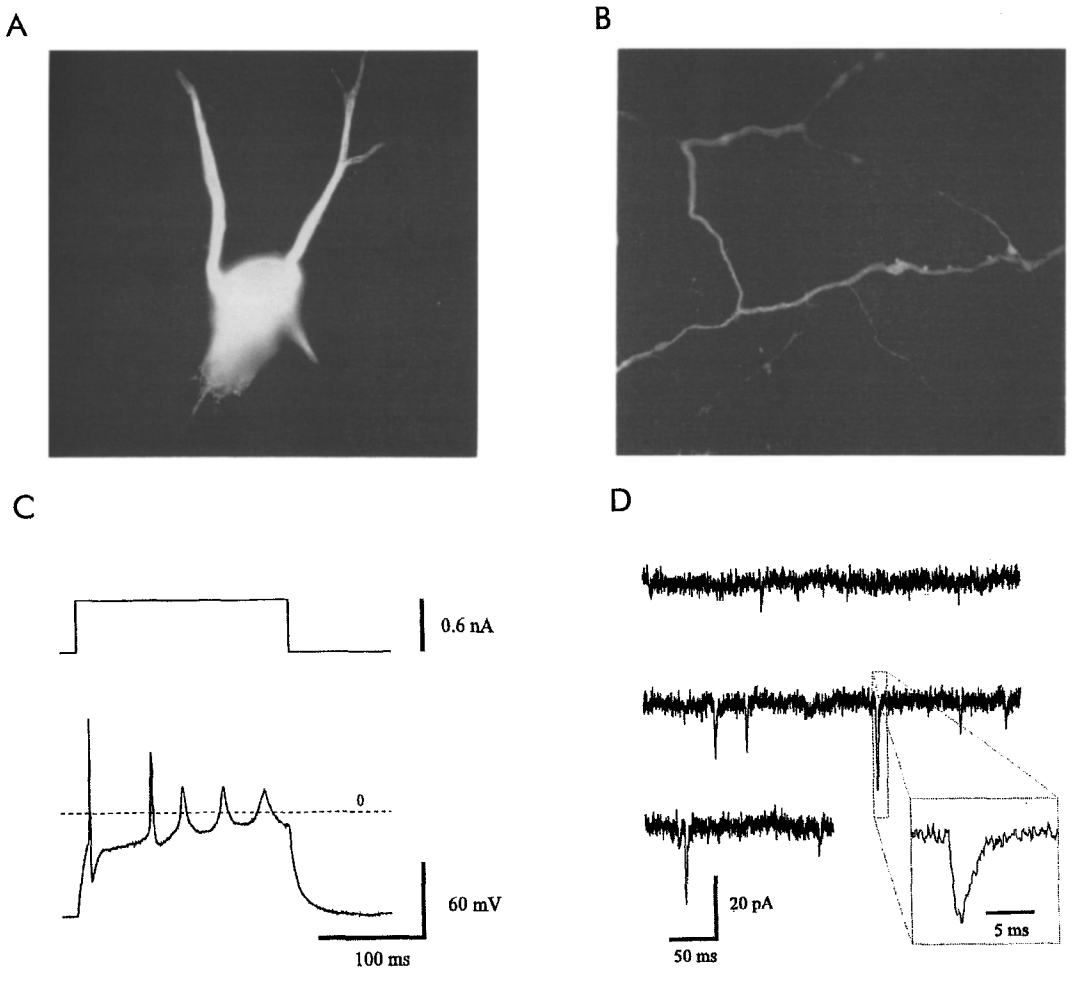

Figure 4. Intrinsic Properties of Retinal Ganglion Cells

Purified RGCs in serum-free B-S cultures containing BDNF, CNTF, high insulin, and forskolin make both dendrites and axons. Cultures were immunostained with an anti-MAP2 monoclonal antibody (A) or an anti-tau monoclonal antibody (B) after 7 days of culture; the primary antibody was detected with a fluorescein isothiocyanate–conjugated anti-mouse IgG antibody. Purified RGCs in the same serum-free culture conditions fire action potentials in response to depolarizing current injection (C) and exhibit spontaneous excitatory postsynaptic currents (D; for illustration, an unusually active time interval is shown).

Effects of Astrocytes and Oligodendrocytes on the Survival of Retinal Ganglion Cells In Vitro

It is not known whether visual pathway glia help promote RGC survival. To find out, we cultured purified P8 RGCs on glass coverslips over a conditioning layer of purified postnatal optic nerve astrocytes (see Experimental Procedures) and measured their survival after 3 days. In serum-free medium containing only forskolin and insulin but no BDNF or CNTF, the astrocytes weakly promoted RGC survival (Table 2). In the presence of BDNF and CNTF, however, there was little further additive effect.

To determine whether oligodendrocytes help promote RGC survival, we similarly cultured purified P8 RGCs over a conditioning layer of immature or mature oligodendrocytes. Immature oligodendrocytes (oligodendrocyte precursor cells) were obtained by sequential immunopanning from P8 optic nerve and were >99.95% pure (Barres et al., 1992); mature oligodendrocytes were prepared by aging the immature oligodendrocytes for 10 days (see Experimental Procedures). In serum-free medium containing forskolin and insulin but no BDNF or CNTF, neither mature nor immature oligodendrocytes promoted RGC survival (Table 2). In the presence of BDNF and CNTF, however, mature oligodendrocytes significantly enhanced RGC survival (Table 2; Figure 5). Under these conditions, the survival of the majority of RGCs could be promoted for at least 1 month, even though they were cultured on glass coverslips (Figure 5). Three lines of evidence suggest that the oligodendrocyte-secreted activity is a protein. It is excluded by filtration of molecules smaller than 10 kDa, and it is destroyed by boiling for 10 min and by trypsin digestion (Table 2). Its additive effect on RGC survival was not mimicked by TGFβ, NGF, or other peptide trophic factors tested.

Table 2. Effects of Glial Cells on the Survival of Purified Retinal Ganglion Cells in Culture

	% Surviving Cells	
Conditioning Cell Type	Forskolin+Ins	Forskolin+BDNF+ CNTF+Ins
Effects of glia (3 DIV)		
None	15.3 ± 0.5	55.0 ± 2.2
Astrocytes	24.5 ± 0.8	54.0 ± 4.0
Immature oligo	17.9 ± 2.1	66.2 ± 4.2
Mature oligo	14.4 ± 0.5	79.0 ± 3.1
Effects of oligo CM (5 DIV)		
No CM		34.4 ± 0.5
Oligo CM		48.8 ± 5.2
Heat-inactivated oligo CM		33.4 ± 1.2
Trypsin-digested oligo CM		26.2 ± 1.0
Oligo CM, fraction >10 kDa		50.2 ± 2.7
Oligo CM, fraction <10 kDa		28.5 ± 0.5

Approximately 15,000 purified P8 RGCs were plated in triplicate onto merosin-coated 12 mm glass coverslips mounted over a conditioning layer of glial cells (see Experimental Procedures) in 24-well Falcon plates in 700 μl of B-S medium containing insulin (5 μg/ml) and forskolin (5 μM), with or without plateau concentrations of BDNF (50 ng/ml) and CNTF (50 ng/ml). After 3 days of culture, the percentage of RGCs surviving in each well was determined using the MTT assay. The survival of RGCs cultured on glass coverslips was generally lower than on plastic, so that these results cannot be directly compared with experiments done on plastic. Approximately 5,000 purified P8 RGCs were plated in triplicate onto merosin-coated wells in a 96-well Falcon plate in 100 μl of B-S medium containing high insulin (5 μg/ml), forskolin (5 μM), BDNF (50 ng/ml), and CNTF (50 ng/ml). The medium was diluted with an equal volume of oligodendrocyte conditioned medium (CM) that had been treated as indicated. DIV, days in vitro.

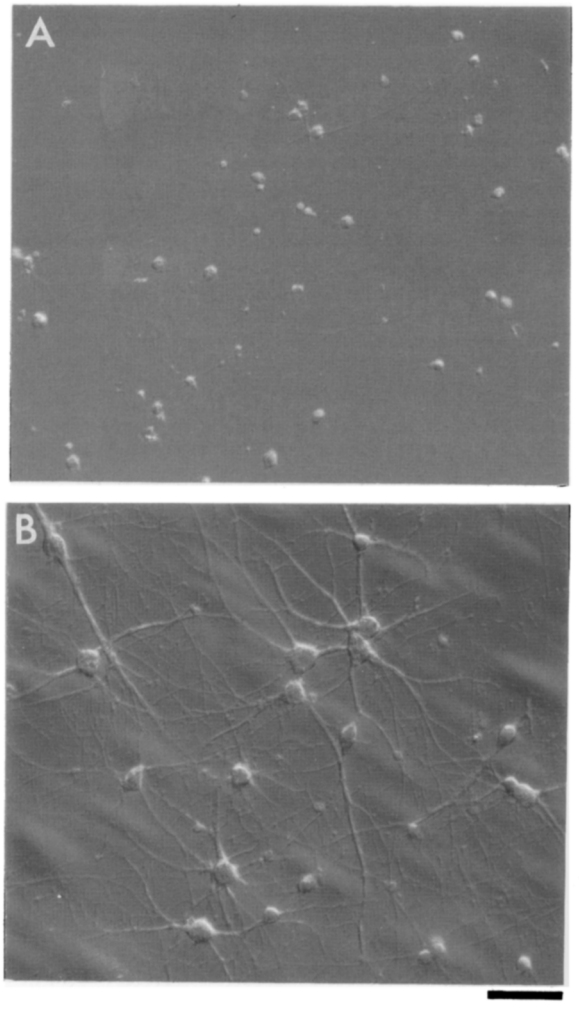

Figure 5. Effects of Oligodendrocyte Conditioned Medium on Postnatal Retinal Ganglion Cell Survival

The morphology of purified P8 RGCs is shown after 14 days in culture without (A) and with (B) a conditioning layer of mature oligodendrocytes. The cells were cultured in serum-free B-S medium containing plateau concentrations of BDNF, CNTF, and IGF1 on merosin-coated glass coverslips. On the glass coverslips in (A), most of the RGCs have died by 2 weeks, but with oligodendrocyte conditioned medium (B), the majority of cells have survived and extended processes. Bar, 40 μm.

Comparison of the Survival Requirements of Embryonic and Postnatal RGCs

To determine whether the signals that promote the survival of P8 RGCs also promote the survival of E18 RGCs, most of which have not yet innervated the tectum, we purified and cultured E18 RGCs in serum-free medium containing various peptide trophic factors. As shown in Figure 6A, BDNF promoted their survival for 3 days as previously reported (Johnson et al., 1986; Rodriguez-Tebar et al., 1989), as did NT-4/5. As was observed for postnatal RGCs, however, no response was observed unless forskolin was present. In contrast to the postnatal RGCs, however, there was little effect of CNTF or oligodendrocyte conditioned medium (Figure 6A). Other factors did not promote survival of purified E18 RGCs, including NGF, NT-3, bFGF, TGFα, TGFβ1, TGFβ2, TGFβ3, LIF, and GDNF, even in combination with forskolin and insulin. Because it has been reported that the responsiveness to merosin differs between embryonic and postnatal RGCs (Cohen and Johnson, 1991), we also tested the effects of a laminin substrate, but the results were similar (data not shown).

Although the embryonic RGCs responded to BDNF, most died after several days as previously reported (Johnson et al., 1986). Neonatal (P1) RGCs, isolated from animals at the peak of RGC cell death, were similar to the E18 cells in that they were also unresponsive to CNTF and most died after 3 days of culture in BDNF. To determine whether visual pathway cells might make other soluble survival signals that act alone or in combination with BDNF, we next studied the effects of conditioned medium from E18 retina, optic nerve, and tectum on the survival of purified E18 RGCs (see Experimental Procedures) in serum-free medium containing plateau levels of BDNF, CNTF, insulin, and forskolin, which were added to promote survival of the conditioning cells. Survival of the E18 RGCs was enhanced nearly 5-fold by soluble tectal factors (Figures 6B and 7).

Effects of Depolarization on the Survival of Retinal Ganglion Cells In Vitro

The requirement for elevation of cAMP for RGCs to respond to peptide trophic factors suggested the possibility that an as yet unidentified signal acts to promote RGC

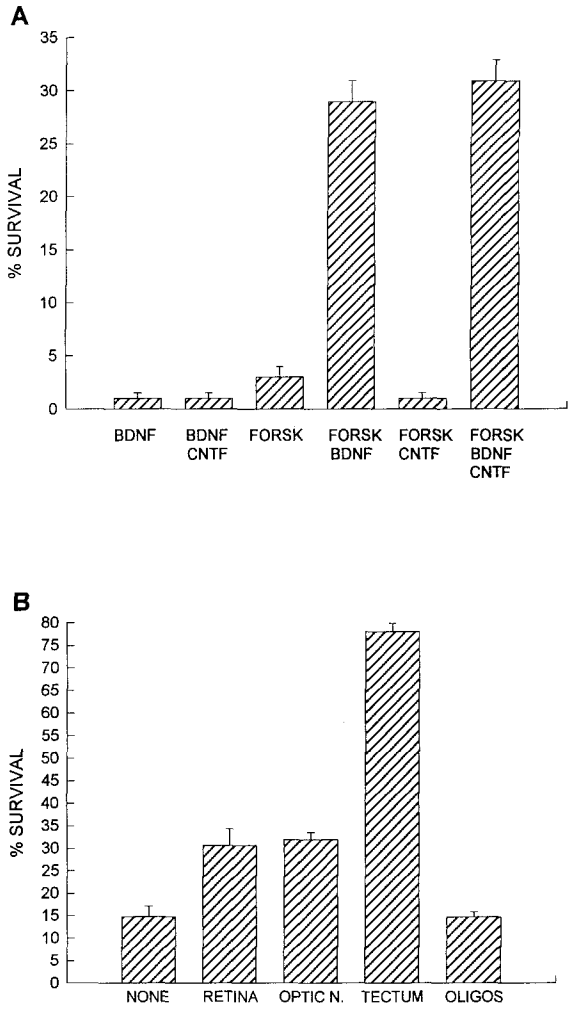

Figure 6. Effects of Survival Factors on Embryonic Retinal Ganglion Cells

(A) The survival of purified E18 RGCs cultured in serum-free B-S medium containing high insulin (5 μg/ml) and various survival factors, as indicated, was assessed after 3 days of culture by the MTT assay.
(B) The survival of purified E18 RGCs after 3 days of culture above a conditioning layer of various cell types in serum-free medium containing forskolin (5 μM) and plateau levels of BDNF, CNTF, and insulin. The conditioning retinal, optic nerve, and tectal cells were isolated from E18 rats, whereas the oligodendrocytes were isolated from P8 rats and matured for 10 days prior to the assay.

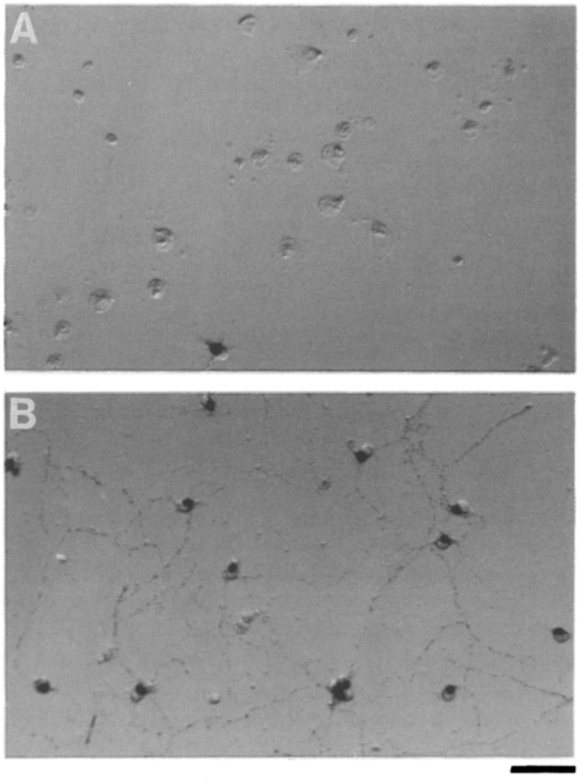

Figure 7. Effects of Tectal Conditioned Medium on Embryonic Retinal Ganglion Cell Survival

The morphology of purified E18 RGCs is shown after 3 days of culture in medium without (A) or with (B) conditioned medium from E18 tectal cells. The cells were cultured in serum-free B-S medium containing plateau concentrations of BDNF, CNTF, and IGF1 on merosin-coated wells in a 96-well plate (A) or on glass coverslips (B). After 3 days, the survival was assessed by the MTT assay. In (A), most of the RGCs have died by 3 days, but with tectal conditioned medium (B), the majority of cells have survived and extended processes. Bar, 30 μm.

survival. Continuous conditioning of medium by retinal, optic nerve, and tectal cells, however, failed to mimic the effect of forskolin, suggesting either that these cell types do not secrete signals that increase RGC cAMP or that desensitization occurred. Because there was little spontaneous synaptic activity in the purified RGC cultures, we wondered whether electrical activity or neurotransmitter stimulation might help the RGCs respond to their trophic factors.

To test this possibility, we added KCl (10–50 mM) to the culture medium. Depolarization alone did not significantly promote RGC survival, but it mimicked the effect of increasing cAMP (Figure 8A). The enhancement of survival induced either by forskolin or depolarization was blocked by the specific protein kinase A inhibitors, Rp-cAMP (100 μM; Figure 8a) (Botelho et al., 1988) and H89 (50 μM; data not shown) (Chijiwa et al., 1990). These inhibitors did not decrease the basal survival in the absence of forskolin (Figure 8B). As a control, we also tested Sp-cAMP (100 μM), the stereoisomer of Rp-cAMP, which mimicked the effect of forskolin or CPT-cAMP as expected. As neither TTX (10 μM) nor kynurenic acid (5 mM) blocks the ability of forskolin to enhance BDNF- and CNTF-promoted survival (Figure 8B), cAMP is probably the distal effector of depolarization.

To determine whether glutamate receptor activation could mimic forskolin, we tested the effects of the glutamate agonists N-methyl-D-aspartate (NMDA; 10 μM), kainate (100 μM), and quisqualate (100 μM) on the survival of purified P8 RGCs cultured in serum-free medium containing BDNF, CNTF, and insulin. The combination of NMDA and either kainate or quisqualate together mimicked forskolin (Figure 8B), and this effect was blocked by Rp-cAMP (and by H89 at 50 μM; data not shown). NMDA, kainate, or quisqualate individually were less effective; the metabotropic agonist APCD had no effect (data not

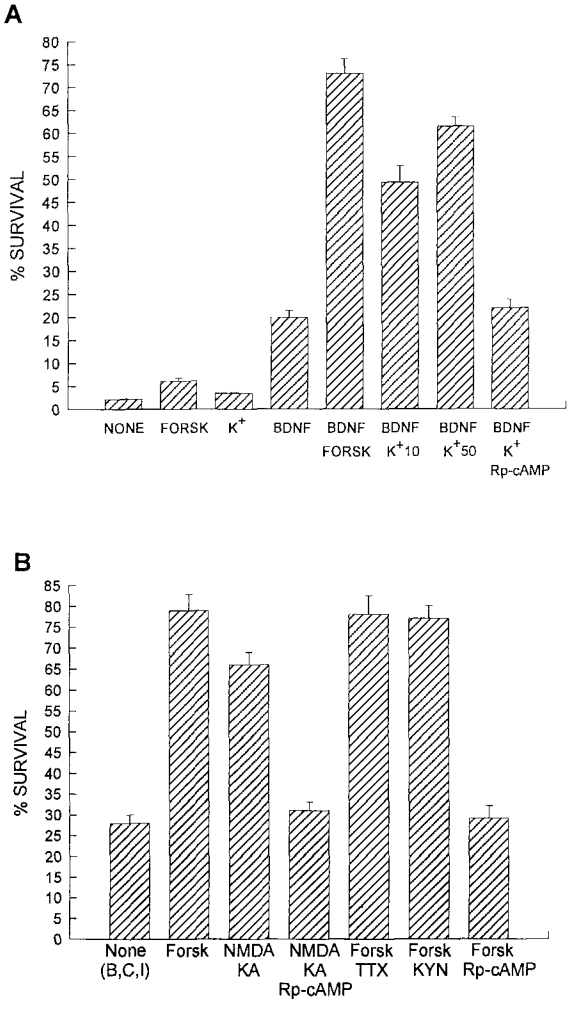

Figure 8. The Effect of Depolarization on Retinal Ganglion Cell Survival

(A) The effect of depolarization induced by K^+. Purified P8 RGCs were cultured in serum-free B-S medium for 2 days in the indicated survival factors. In some cases, either forskolin (5 μM) or KCl (10 or 50 mM as indicated; when not indicated, the results for 10 and 50 mM were nearly identical) was also included in the medium. Rp-cAMP is a specific inhibitor of protein kinase A.

(B) The effects of glutamate agonists. Purified P8 RGCs were cultured in serum-free medium for 3 days in BDNF, CNTF, and insulin. The effects of adding forskolin (5 μM), TTX (10 μM), kynurenic acid (5 mM), NMDA (10 μM), and kainate (100 μM) on survival are shown.

shown). To our surprise, we observed no evidence of excitotoxicity of developing RGCs even after 3 days of culture in high (100 μM) concentrations of NMDA, kainate, and quisqualate together.

Discussion

Multiple Signaling Molecules Collaborate to Promote the Long-Term Survival of Retinal Ganglion Cells In Vitro

These results demonstrate that highly purified embryonic and postnatal RGCs are unable to produce signals sufficient to promote their own survival, even when cultured at high density, suggesting that their survival depends on signals from other cell types. Peptide trophic factors that have previously been shown to promote short-term RGC survival in vitro include BDNF (Johnson et al., 1986; Lehwalder et al., 1989) and NT-4/5 (Cohen et al., 1994). In vivo, adult RGC survival after optic nerve transection can be promoted by intraocular injection of BDNF (Mansour-Robaey et al., 1994), CNTF (Mey and Thanos, 1993), and FGF (Sievers et al., 1987). Our results directly demonstrate that these and several other peptide factors act directly on postnatal RGCs to promote their survival. Furthermore, they show that long-term survival of RGCs in culture can be promoted by a combination of peptide trophic factors normally produced by their neighboring cell types along the visual pathway. These signals include BDNF, which is made by the tectum (Sievers et al., 1987), CNTF or LIF, which are made by optic nerve astrocytes and tectum (Stockli et al., 1991; Yamamori, 1991; Patterson and Fann, 1992), and IGF1, which is made by astrocytes (Ballotti et al., 1987; Hannson et al., 1989; Rotwein et al., 1988; Baron-van Evercooren et al., 1991), in addition to an unknown protein made by oligodendrocytes. We have so far identified seven peptide factors (BDNF, NT-4/5, CNTF, LIF, IGF1, bFGF, and TGFα) that can promote RGC survival, and our findings suggest there are at least two others remaining to be identified, one from oligodendrocytes and one from tectum. The matrix proteins laminin and merosin promote RGC survival, as well as enhance their neurite outgrowth, as previously reported (Cohen and Johnson, 1991).

The ability of collaborative signaling by multiple trophic factors to promote long-term neuronal survival is not unique to RGCs. The long-term survival of oligodendrocytes and of spinal motor neurons also appears to be enhanced in the presence of multiple peptide factors (Arakawa et al., 1990; Barres et al., 1993a; Mitsumoto et al., 1994). RGCs have been divided into several subsets of cells that differ in morphology and electrophysiological properties (Boycott and Wassle, 1974; Perry, 1979; Perry and Walker, 1980). However, it is unlikely that the requirement for multiple trophic factors reflects the presence of multiple subsets of cells each with different trophic factor sensitivities, as the long-term survival responses to single factors did not add to produce the survival values seen with multiple factors together (see Figure 1C). Thus, our data strongly suggest that multiple factors act collaboratively on individual RGCs.

Our data do not exclude the possibility that an as yet unidentified molecule exists that is sufficient to promote RGC survival by itself. A dependence of CNS neurons on multiple survival factors, however, might help them to develop their complex patterns of afferent and efferent connections (Snider, 1994). In principle, a dependence on multiple survival factors could explain how RGCs establish topographically correct connections if these factors are distributed in graded concentrations along opposing directions in the tectum (the data of Simon and O'Leary [1992] are suggestive of this possibility, as they show that the retinotectal map is formed during the period of normal RGC death).

Oligodendrocytes Promote Survival of Postnatal RGCs

The possibility that oligodendrocytes help to promote neuronal survival has received little attention, although other types of glial cells (including cortical astrocytes and Schwann cells) have been clearly shown to help promote RGC survival in vitro (McCaffery et al., 1984, 1985; Raju and Bennet, 1986; Armson et al., 1987; Kleitman et al., 1988; Baehr and Bunge, 1990; Maffei et al., 1990; Baehr et al., 1991; Thanos et al., 1989). Except for TGFβ and NGF, which do not promote RGC survival, oligodendrocytes have not been found to express trophic factors (McKinnon et al., 1993; Byravan et al., 1994). Our findings demonstrate that oligodendrocytes secrete a soluble protein with a molecular mass greater than 10 kDa whose trophic activity is not mimicked by known factors we have tested. It additively promotes the long-term survival of postnatal RGCs cultured in medium containing BDNF, CNTF, and IGF1, although it is unable to promote survival by itself. It is likely that optic nerve glia promote RGC survival in vivo because the time course of RGC death is delayed when the optic nerve is transected near the chiasm instead of retroorbitally (Villegas-Perez et al., 1992; Berkelaar et al., 1994).

Just as oligodendrocytes help to promote RGC survival, we have previously found that RGCs are essential for long-term survival of oligodendrocytes. When axons degenerate after optic nerve transection, oligodendrocytes undergo apoptosis (Barres et al., 1993b). In normal animals, this reciprocal trophic interaction should stabilize the survival of both RGCs and their oligodendrocytes; after axotomy, however, both RGCs and oligodendrocytes die. Loss of oligodendrocyte-derived survival factors could help to explain the failure of CNS neurons to survive and regenerate after transection.

The Survival Requirements of Retinal Ganglion Cells Change around the Time of Target Innervation

The survival requirements of highly purified E18 and P8 RGCs are very different (Table 3). CNTF, LIF, FGF, TGFα, and the oligodendrocyte factor helped to promote the survival of the postnatal but not the embryonic RGCs. Together, BDNF, CNTF, insulin, and forskolin promoted the long-term survival of P8 RGCs, but only the short-term survival of the E18 (and P1) RGCs. Most RGCs innervate the tectum between E18 and P4, and the survival requirements of RGCs appear to change at about this time. This change could be caused by intrinsic aging, endocrine signals, or target-derived signals. Similarly, the responsiveness of RGCs to laminin and merosin has previously been shown to change around the time of target innervation (Cohen and Johnson, 1991; de Curtis et al., 1991).

Our findings point to the crucial importance of tectal signals in promoting the survival of RGCs at about the time of target innervation, as previously suggested (Rodriguez-Tebar et al., 1989). Neurotrophins alone, however, are insufficient to account for these tectal signals. BDNF (or NT-4/5) alone or together with other known factors was sufficient only to promote short-term survival of about 20% of the purified embryonic RGCs; NGF and NT-3 did not promote their survival at all. In contrast, nearly 80% of the E18 RGCs were promoted by BDNF together with tectal conditioned medium. These results demonstrate that the tectum secretes a significant survival activity that cannot be mimicked by any known neurotrophin, including NGF, BDNF, NT-3, and NT-4/5. A chondroitin sulfate proteoglycan purified from tectum has recently been reported to promote neonatal RGC survival in culture (Schulz et al., 1990); it seems unlikely that this molecule alone accounts for the tectal activity we observed since it promotes RGC survival for only about a day.

Table 3. Effects of Survival Factors on Embryonic and Postnatal Retinal Ganglion Cells

Factor	Embryonic RGCs	Postnatal RGCs
Require cAMP or depol.	Yes	Yes
BDNF (or NT-4/5)	Yes	Yes
E18 tectal factor	Yes	ND
CNTF	No	Yes
LIF	No	Yes
bFGF	No	Yes
TGFα	No	Yes
RGC paracrine factor	No	Yes
Oligodendrocyte factor	No	Yes

The table summarizes observations from the present paper; in all cases, the E18 or P8 RGCs were cultured in serum-free B-S medium containing forskolin (5 μM) and were >99.5% pure.

Together with previous findings, our results suggest a simple model for how RGC survival is controlled during the development of the retinotectal projection. At the time of target innervation, embryonic RGCs may survive primarily in response to tectum-derived peptide factors, including BDNF and an as yet unidentified tectal signal. A competition for limiting amounts of tectal signals could help select which RGCs survive and which die, as previously proposed (Rodriguez-Tebar et al., 1989). Survival of the selected neurons may then be stabilized in the postnatal animal by glia-derived survival factors. Most glial cells develop after target innervation; astrocytes could then provide IGF1, CNTF or LIF, and possibly BDNF or NT-4/5 as well (Zafra et al., 1992), and oligodendrocytes could provide their factor. In addition, after target innervation, autocrine or paracrine mechanisms may be initiated, such as FGF production by the RGCs (although this autocrine factor does not appear to be bFGF, another FGF family member remains a possibility). This model is similar to a recently proposed model of PNS sensory neuron development (Acheson et al., 1995; Heymach and Barres, 1995), although in the case of RGCs, autocrine signaling alone is insufficient to promote their survival. An important question is whether the adult tectum continues to provide survival signals to postnatal RGCs or whether they come to depend entirely on glial cells.

Elevation of cAMP Is Necessary for Retinal Ganglion Cells to Respond to Their Survival Signals

An important finding in the present study is that purified embryonic and postnatal RGCs, in contrast to other types of purified neurons previously studied, exhibited almost no responsiveness to peptide trophic factors unless their

intracellular cAMP was simultaneously elevated. K^+-induced depolarization or glutamate receptor activation mimicked the effects of forskolin (or of CPT-cAMP), and their effects were blocked by protein kinase A inhibition, strongly suggesting that they act by elevating intracellular cAMP. Similarly, elevation of cAMP by depolarization has been reported to promote trophic factor responsiveness in several neuronal cell lines (Birren et al., 1992; Mark et al., 1995; Reddy et al., 1995). We do not yet know whether physiological electrical activity consisting of trains of action potentials, rather than chronic depolarization, will enhance the survival of RGCs in culture. Electrical activity does, however, increase cAMP levels in hippocampal neurons (Frey et al., 1993).

Both electrical activity and elevation of cAMP have previously been reported to enhance RGC survival in mixed retinal cultures (Lipton, 1986; also see Jackson et al., 1982; Brenneman et al., 1985; Brenneman and Eiden, 1986). As this effect was mimicked by vasoactive intestinal peptide, whose receptors are located on Muller glia, the effect of cAMP was suggested to be mediated indirectly by enhanced glial trophic factor release (Lipton, 1986; Kaiser and Lipton, 1990). The present results demonstrate that increasing intracellular cAMP and depolarization act directly on RGCs in vitro to enhance their survival by enhancing their responsiveness to peptide trophic factors. Several previous studies suggest that electrical activity may also help to control RGC survival and development in vivo. Developing RGCs are electrically active (Maffei and Galli-Resta, 1990; Meister et al., 1991), and electrical activity and glutamate receptor activation affect the survival and growth of developing RGCs and their processes (Fawcett et al., 1984; Shatz and Stryker, 1988; Simon et al., 1992; Bodnarenko and Chalupa, 1993; O'Rourke et al., 1994). Although Fawcett et al. (1984) found that intraocular injections of TTX into developing retina did not decrease the total number of RGCs that survived, TTX would not be expected to block glutamatergic or other depolarizing afferent neurotransmitter stimulation occurring within the retina.

How does elevation of cAMP enhance the responsiveness of RGCs to many different peptide factors, including BDNF, CNTF, LIF, IGF1, TGFα, and FGF? cAMP could enhance the production or release of an autocrine or paracrine trophic factor (Ghosh et al., 1994) that acts collaboratively with these peptide factors. A second possibility, which we favor because cAMP elevation was effective at clonal density, is that cAMP enhances the sensitivity of RGCs to these peptide factors, either by increasing the number of their receptors (Birren et al., 1992) or by potentiating their signal transduction pathways (Sheng et al., 1990; Ghosh and Greenberg, 1995; Robertson et al., 1995). We do not yet know, however, whether the ability of cAMP to promote RGC survival is transcriptionally dependent.

Do CNS and PNS Neurons Have Different Survival Requirements?

Our findings raise the possibility that the fundamental mechanisms that control the survival of CNS and PNS neurons may differ. First, in contrast to the RGCs, the survival of highly purified PNS neurons, such as sensory, sympathetic, and ciliary neurons, can be stimulated by single peptide trophic factors (Barde, 1989) in the absence of depolarization or elevated cAMP. Although elevated cAMP can synergistically enhance the effects of NGF on PC12 cells (Gunning et al., 1981), a model PNS neuron, most effects of NGF on PC12 cells are not blocked by protein kinase A inhibition (Ginty et al., 1991). Second, also in contrast to the RGCs, elevation of cAMP or depolarization promotes the short-term survival of many PNS neurons by inducing growth factor independence (Wakada et al., 1983; Rydell and Greene, 1988; Koike and Tanaka, 1991; Franklin and Johnson, 1992).

The survival of other types of CNS neurons in vivo is also enhanced by electrical activity (Catsicas et al., 1992; Galli-Resta et al., 1993). In preliminary experiments, we have isolated and cultured highly purified populations of cerebral cortical neurons and found that elevation of cAMP is essential for their responsiveness to BDNF and CNTF. Thus, the survival requirements of RGCs, and at least some other CNS neurons, appear to be different from those previously reported for PNS neurons, as they exhibit little responsiveness to their trophic factors unless their intracellular cAMP is elevated.

A cAMP-dependent mechanism that enhances trophic signals coinciding with activity has important implications because it should reward the survival of active over silent neurons. This mechanism could help to promote the survival and growth of processes by active visual neurons during development (Shatz and Stryker, 1988). In other CNS regions, it might participate in the activity-dependent formation of new synaptic connections during learning (Frey et al., 1993). Finally, it might help explain why PNS neurons are better able to regenerate than CNS neurons: injured, less active RGCs may fail to survive and regenerate after axotomy not only because they do not obtain needed survival signals but also because they have impaired responsiveness to their survival signals.

Experimental Procedures

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

Reagents

Recombinant human IGF1, bFGF, TGFα, and TGFβ were obtained from Peprotech (NJ). Insulin was obtained from Sigma (MO). Recombinant trophic factors were generously provided by the following colleagues: Yves Barde (mouse NT-3), Regeneron (human BDNF and NT-4/5), Michael Sendtner and Hans Thoenen (rat CNTF), and John Heath (human LIF).

Preparation of Retinal, Optic Nerve, and Tectal Suspensions

The tissue, either E18 or P8 retinas or neonatal optic nerves, was obtained from S/D rats (Simonsen Labs, CA) and dissociated enzymatically to make a suspension of single cells, essentially as described by Huettner and Baughman (1986). In brief, the tissue was incubated at 37°C for 30 min in a papain solution (15 U/ml for retina or 30 U/ml for optic nerve; Worthington) in an Earle's balanced salt solution (EBSS, Gibco) containing L-cysteine. The tissue was then triturated

sequentially (retina with a 1 ml pipette, optic nerve with #21 and then #23 gauge needles) in a solution containing ovomucoid (2 mg/ml; Boehringer-Mannheim), DNase (0.004%; Sigma), and bovine serum albumin (BSA; 1 mg/ml; Sigma) to yield a suspension of single cells. After centrifugation at 800 × g, the cells were rewashed in another ovomucoid/BSA solution (10 mg/ml each). To prepare neonatal tectal suspensions, the tecta were incubated in an EBSS solution containing trypsin (0.025%; Sigma) and DNase (0.004%) for 15 min at 37°C. The tissue was then triturated into a cell suspension by passing it repetitively through a 1 ml pipet in a 10% fetal calf serum solution.

Purification of Retinal Ganglion Cells

RGCs from E18 or P8 S/D rats were purified essentially as previously described (Barres et al., 1988). Using sequential immunopanning, RGCs were isolated to >99.5% purity. Typically, about 20% of the RGCs were isolated, which is about 40,000 RGCs per P8 animal. A brief description of the procedure follows.

Preparation of Panning Plates

Secondary antibodies included affinity-purified goat anti-mouse IgM (μ chain–specific; Jackson) and affinity-purified goat anti-rabbit IgG (H + L chain–specific; Jackson). Primary antibodies included monoclonal supernatant IgM antibody against mouse Thy1.1 (T11D7e2, American Type Culture Collection, TIB 103). Petri dishes (two 150 × 15 mm and one 100 × 15 mm; Fisher) were incubated with 5–15 ml of Tris buffer solution (pH 9.5) with 10 μg/ml secondary antibody (anti-mouse IgM on the small dish or anti-rabbit IgG on the large dishes) for 12 hr at 4°C. The dishes were washed three times with 8 ml of phosphate-buffered saline (PBS), and the small dish was further incubated with 5 ml of Thy 1.1 IgM monoclonal supernatant for 2 hr at room temperature. The supernatant was removed, and the plate was washed three times with PBS. To prevent nonspecific binding of cells to the panning dish, 5–15 ml of PBS with 2 mg/ml BSA was placed on the plates for 20 min.

Panning Procedure

The retinal suspension was incubated in anti-rat-macrophage antiserum (Axell; 1:100) for 20 min, centrifuged, resuspended in PBS, and incubated on a 150 mm anti-rabbit IgG panning plate at room temperature for 45 min. The plate was gently swirled every 15 min to ensure access of all cells to the surface of the plate. If cells from more than eight retinas were panned, the nonadherent cells were transferred to another 150 mm anti-rabbit IgG panning plate for another 30 min. The nonadherent cells were removed with the suspension, filtered through 15 μm Nitex mesh (Tetko), and placed on the T11D7 panning plate. The cells were incubated on the plate as described above. After 45 min, plates were washed eight times with 10 ml of PBS and swirled moderately vigorously to dislodge nonadherent cells. Progress of nonadherent cell removal was monitored under the microscope. The washing was terminated when only adherent cells remained.

Removing Adherent Cells from the Plate

Four milliliters of a trypsin solution (0.125%) was prepared by diluting a trypsin 20× stock (Sigma) into EBSS. Cells on the panning dish were incubated with this solution for 10 min in a 10% CO_2 incubator at 37°C. The cells were dislodged by gently pipetting trypsin solution around the plate. Ten milliliters of a 25% fetal calf serum solution were added to inactivate the trypsin, and the cells were spun and collected as above.

Purification of Optic Nerve O-2A Progenitor Cells, Oligodendrocytes, and Astrocytes by Sequential Immunopanning

Oligodendrocytes and their precursors were purified from the optic nerves of P8 S/D rats, as previously described (Barres et al., 1992). A brief description of the procedure follows.

Preparation of Panning Plates

Secondary antibodies were affinity-purified goat anti-mouse IgM (μ chain–specific) and affinity-purified goat anti-mouse IgG (H + L chain–specific). Primary monoclonal antibodies were the A2B5 antibody (IgM; Eisenbarth et al., 1979), anti-RAN-2 antibody (IgG; Bartlett et al., 1981), and anti-GC antibody (IgG; Ranscht et al., 1982). Petri dishes (100 × 15 mm; Falcon) were incubated with 10 ml of Tris buffer solution (50 mM; pH 9.5) containing 10 μg/ml secondary antibody, either anti-IgM or anti-IgG, for 12 hr at 4°C. Each dish was washed three times with 8 ml of PBS and incubated with 5 ml of either A2B5 ascites at 1:2000 (one IgM dish), anti-RAN-2 supernatant at 1:4 (two IgG dishes), or anti-GC supernatant at 1:4 (one IgG dish) for at least 1 hr at room temperature. The antibodies were diluted in PBS containing BSA (1 mg/ml; Sigma A4161) in order to block the nonspecific adherence of cells to the panning plates. The antibody solution was removed, the plates washed were three times with PBS, and PBS was left on the plates until use.

Immunopanning Procedure

The optic nerve cell suspension was resuspended in 7 ml of PBS containing insulin (5 μg/ml; Sigma) and filtered through Nitex mesh (15 μm pore size; Tetko). To deplete type 1 astrocytes and meningeal cells (as well as microglia and macrophages, which stick via their Fc receptors to the first immunoglobulin-coated panning dish used), the cell suspension was first placed on the RAN-2 plate for 30 min at room temperature, with brief and gentle agitation after 15 min. The nonadherent cells were transferred to the second RAN-2 plate for 30 min, after which the nonadherent cells were transferred to the A2B5 dish to deplete the O-2A progenitor cells and newly formed oligodendrocytes. After 45 min, this plate was washed eight times with 10 ml of PBS, with moderately vigorous agitation in order to remove the nonadherent cells. The progress of nonadherent cell removal was monitored under an inverted phase-contrast microscope, and washing was terminated when only adherent cells remained.

The purification procedure for optic nerve type 1 astrocytes follows a similar sequential immunopanning protocol with a few differences. Optic nerves from P2 rats are used. The first panning dish is coated with the MRC-OX7 Thy1.1 antibody to deplete the suspension of meningeal fibroblasts, the second dish is coated with the A2B5 and GC antibodies to deplete the suspension of O-2A lineage cells, and the final dish is coated with the C5 monoclonal antibody (Miller et al., 1984) to select any remaining neuroepithelial cells, which are >99.5% GFAP-positive type 1 astrocytes.

Culture of Purified Retinal Ganglion Cells

Approximately 5,000 purified RGCs were cultured in 96-well plates (Falcon) that had been coated with poly-D-lysine (70 kDa; 10 μg/ml; Sigma) followed by merosin (2 μg/ml; Telios/GIBCO) in 100 μl of a serum-free medium containing Neurobasal, a recently described medium that has been optimized for neuronal cell culture (Brewer et al., 1993). The use of Neurobasal instead of Dulbecco's modified Eagle's medium (DMEM) was essential for good survival of the purified RGCs. DMEM and Neurobasal differ in several ways; the most essential in this case was its lower osmolarity of 235 mOsm instead of the 335 mOsm of DMEM (the final osmolarity of our culture medium when the serum-free additives were included was about 260 mOsm). The serum-free additive included BSA, selenium, putrescine, thyroxine, tri-iodothyronine, transferrin, and progesterone (modified from Bottenstein and Sato, 1979, as previously described in Lillien and Raff, 1990) (B-S medium), pyruvate (1 mM), glutamine (1 mM), and the appropriate trophic factors. The B-S medium was prepared with a highly purified, crystalline grade of BSA (A4161, Sigma) to avoid contaminating survival factors. Signaling molecules present in the B-S serum-free medium, particularly progesterone, tri-iodothyronine, and thyroxine, are important contributers to RGC survival (unpublished data). The percentage of surviving cells was assessed after 3, 7, and 14 days by the MTT assay (see below). All values were normalized to the percentage of surviving cells at 3 hr after plating, which represented the percentage of cells that survived the purification procedure (we have observed that the initial viability of cells purified by panning is always about 85%, regardless of the type of cell being panned, with the 15% of dead cells counted in the asssessment of initial viability being clearly necrotic and thus morphologically different from the cells that later die, which have a shrunken morphology characteristic of apoptosis).

Preparation of Cocultures and Conditioned Medium

To assess the RGC survival effects of soluble factors released by retinal cells, tectal cells, optic nerve cells, or purified astrocytes or oligodendrocytes, we plated about 50,000 cells onto the bottom of poly-D-lysine- or merosin-coated wells in a 24-well plate and allowed the cells to condition serum-free Sato medium, which did or did not contain additional trophic factors depending on the experiment as described, for at least 3 days. In some cases, purified oligodendrocytes

were allowed to mature for 10 days prior to further use. After 3 days, three small sterile glass chips were added to the bottom of each well to act as pedestals to support a 13 mm merosin-coated glass coverslip. The glass coverslip was added to each well, and 10,000 purified RGCs were plated onto each slip. Thus, each well contained two layers of cells that did not contact each other, with the conditioning cells on the bottom layer and the purified RGCs on the top layer. The medium was changed by replacing half of the volume of the well with fresh medium every 3 days.

Characterization of the Oligodendrocyte-Derived RGC Survival Factor

Serum-free Sato medium containing CNTF (50 ng/ml) and high insulin (5 μg/ml) was conditioned by a dense culture of mature oligodendrocytes for at least 3 days. To determine whether the non-CNTF, non-insulin survival activity was a protein, we heat inactivated the conditioned medium by boiling for 5 min. We also incubated the medium for 16 hr at 37°C with 0.5 ml of a solution of trypsin-coupled beads (Pierce). As a control, medium lacking beads was incubated in parallel. The trypsin digestion was terminated by centrifugation at 1000 × g for 10 min to separate the beads from the supernatant. The ability of the heat-inactivated, trypsin-digested medium, diluted 1:1 with fresh nontreated, serum-free unconditioned medium, to promote survival was assessed on purified RGCs and compared with control medium. To assess whether the size of the unknown factor was greater than 10 kDa, we centrifuged the oligodendrocyte conditioned medium four times through a Centriprep-10 concentrator membrane (Amicon), which excludes molecules greater than 10 kDa, at 2500 × g for 50 min. The medium was concentrated by 10-fold. The ability of a 20-fold dilution of the concentrated medium (containing activities greater than 10 kDa) and a 1:1 dilution of the filtrate (containing activities less than 10 kDa) to promote survival was assessed.

MTT Survival Assay

The MTT survival assay was performed as described by Mosmann (1983). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in PBS at 5 mg/ml and sterilized by passage through a Millipore filter (0.22 μm). This stock solution was added to the culture well (1:9) and incubated at 37°C for 1 hr. Viable cells with active mitochondria cleaved the tetrazolium ring into a visible dark blue formazan reaction product. The viable and dead cells in each well were counted by bright-field microscopy. In all cases, the percentage survival determined by the MTT assay was nearly identical to the values determined by morphology alone. All values are given as the mean ± SEM of at least three cultures. All experiments were repeated at least three times. The results of representative experiments are shown. Statistical significance was determined by the unpaired Student's t test. Significance was assumed for all reproducible values of $p < .05$.

Immunofluorescence Staining

After fixation with 4% paraformaldehyde for 10 min at room temperature, cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 mM L-lysine to block nonspecific binding and 0.4% Triton X-100 to permeabilize the membrane. To stain the cytoskeletal proteins in dendrites and axons, cells were incubated in monoclonal anti-MAP2 antibody (5 μg/ml; Boehringer-Manheim) or in monoclonal anti-tau antibody (5 μg/ml; Boehringer-Manheim), respectively, followed by fluorescein-coupled goat anti-mouse IgG (10 μg/ml; Jackson). The coverslips were mounted in Citifluor on glass slides, sealed with nail varnish, and examined in a Zeiss Axioskope fluorescence microscope.

Electrophysiological Recording

Standard procedures for preparing pipets, seal formation, and whole-cell patch-clamp recording using an Axopatch 200A patch clamp were utilized. Patch pipets were pulled from hard borosilicate capillary glass (WPI) and had resistances of about 3–10 MΩ. All experiments were performed at room temperature, ~ 24°C. The bath solution contained 120 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM KCl, and 10 mM HEPES (pH 7.3); the pipet solution contained 100 mM K-gluconate, 10 mM KCl (Ca^{2+}-buffered to 10^{-6} M with 10 mM EGTA), and 10 mM HEPES (pH 7.3).

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