Activation of MAPK and CREB by GM1 Induces Survival of RGCs in the Retina with Axotomized Nerve

Jun-Sub Choi,[1] *Jeong-a Kim,*[2] *and Choun-Ki Joo*[1]

Purpose. Neuronal cells undergo apoptosis when the supply of neurotrophic factor is limited by injury, trauma, or neurodegenerative disease. Ganglioside has both neuritogenic and neurotropic functions. Exogenously administered monosialoganglioside (GM1) has been shown to have a stimulatory effect on neurite outgrowth and to prevent degeneration of neuronal cells in the central nervous system. Even though GM1 has been shown to mimic, or have synergy with, neurotrophic factors, the neuroprotective mechanism of GM1 has not been well understood. In this study, optic nerve transection, or axotomy, was used as an in vivo model system for injury, to examine the protective mechanism of GM1 in injured retinal ganglion cells.

Methods. GM1 was injected into the vitreous body before axotomy, and the protective effect of GM1 observed with regard to activation of mitogen-activated protein kinase (MAPK) and phosphorylation of cAMP-responsive element–binding (CREB) protein. Activation of MAPK and CREB were examined by Western blot analysis and immunohistochemistry, and the surviving retinal ganglion cells were counted after retrograde fluorescence labeling.

Results. GM1 inhibited the degeneration of axotomized retinal ganglion cells. In addition, GM1 enhanced the activation of MAPK and CREB with the treatment of GM1 in the retina with axotomized nerve. Treatment of MAPK inhibitor PD98059 with GM1 reduced the protective action of GM1 and prevented GM1-induced phosphorylation of CREB.

Conclusions. GM1 protected the axotomized retinal ganglion cells (RGCs) from cell death after axotomy through the activation of MAPK and CREB. (*Invest Ophthalmol Vis Sci.* 2003;44:1747–1752) DOI:10.1167/iovs.01-0886

Neuronal cell death is caused by withdrawal of neurotrophic factors or as a consequence of acute insults, such as ischemia, trauma, and neurodegenerative disease.[1] Therapeutic trials have been performed to determine the effectiveness of various agents in preventing neuronal cell death in various diseases and injuries. In addition to neurotrophic factors,[2–4] there is a growing number of substances that block neuronal cell death. Monosialoganglioside (GM1) is one of the substances intensively evaluated for use as a therapy for neurodegenerative disease.[5–7]

GM1 is a naturally occurring sialic acid containing glycosphingolipid and is a component of the plasma membrane in eukaryotic cells.[8] Relatively high proportions (5%–10%) of total membrane lipids are composed of sialic acid–containing glycosphingolipids in neuronal cells.[8] Gangliosides play a role in controlling the rigidity of membrane and are involved in cell–cell communication, neuronal development, and differentiation.[7,9] GM1 has been reported to have a neuroprotective function in cerebral ischemia[7,10] retinal culture models of excitotoxicity,[7,11] as well as in neuronal apoptosis.[6,12–15] Either the potentiating or mimicking of neurotrophic factors is postulated to be the mechanism of neuroprotective action.[6,16–18] Previous reports showed that GM1 potentiates autophosphorylation and dimerization of Trk family receptors for neurotrophic factors (nerve growth factor [NGF], brain derived neurotrophic factor [BDNF], NT4/5).[13,16,19,20] The neuroprotective mechanism of GM1 seems to occur through the activation of mitogen-activated protein kinase (MAPK)[21] and/or the phosphatidylinositol 3-kinase (PI 3-K) pathway.[12] Furthermore, inhibition of ganglioside synthesis prevents the NGF-mediated activation of MAPK and PI 3-K.[22]

One of the well-known target molecules of MAPK and PI 3-K is cAMP response element binding (CREB) protein.[23] CREB seems to be the key modulator of a general intrinsic survival program.[24,25] A previous report showed that CREB enhances the survival of PC12 cells by promoting the expression of *bcl-2*.[25]

Optic nerve (ON) transection (axotomy) causes irreversible degeneration of retinal ganglion cells (RGCs). After axotomy, ganglion cells ultimately die due to deprivation of neurotrophins, altered gene expression, and various reactive oxygen species.[26–30] There have been numerous studies on the protection of the retina that involved application of various neuroprotective reagents to the retina with axotomized nerve.[28,31–36] In fact, intraocular administration of neurotrophic factors (BDNF, NGF, and NT3/4) enhances the survival of RGCs.[36,37]

Even though there have been many reports showing the neuroprotective effect of GM1,[6,9,19,38] the neuroprotective mechanism of GM1 in neuronal degeneration has not been studied in relation to the phosphorylation status of CREB. In this study, we observed activation of MAPK and CREB in the retina with axotomized nerve, with and without GM1. Also, we investigated the relation of MAPK and CREB in a GM1-mediated neuroprotective mechanism.

Material and Methods

Animals and Axotomy

Adult male Sprague-Dawley rats (200–250 g) were kept in a 12-hour light–dark cycle. Chloral hydrate (400 mg/kg) was used for anesthesia. All rats were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The optic nerve was transected approximately 5 mm from the posterior pole of the eye, by using an intraorbital approach guided by a surgical microscope. The retinal blood flow was then checked by direct stereomicroscopy, and

From the [1]Department of Ophthalmology and Visual Science, College of Medicine, The Catholic University of Korea, Seoul, Korea; and the [2]Diabetes Unit, National Center for Complementary and Alternative Medicine, National Institutes of Health, Bethesda, Maryland.

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Corresponding author: Choun-Ki Joo, Department of Ophthalmology and Visual Science, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-ku, Seoul 137-701, Korea; ckjoo@cmc.cuk.ac.kr.

those animals with normal blood supply and cleared lens were used in the experiment. GM1 was dissolved in 0.9% sodium chloride solution and stored at 4°C. Before injection of GM1, in each eye, the aqueous humor was removed from the anterior chamber by suction with a 30-gauge needle through a corneal puncture. Ten microliters of 2 mM GM1 was injected into the vitreous body with a syringe (Hamilton, Reno, NV), under a surgical microscope (Olympus, Tokyo, Japan), 10 minutes before axotomy. Ten microliters of the MAPK inhibitor PD98059 (50 μM; Calbiochem, Darmstadt, Germany) was injected before injection of GM1. Intravitreous injection was performed from limbus to vitreous body without damage to the lens. Five animals were used in each group. In the control group for GM1 and PD98059, 10 μL saline was injected into the vitreous body. After the vitreous injection, no acute inflammation was observed in the retina.

Immunohistochemistry

Eyes were fixed for 2 hours in 4% paraformaldehyde and then corneas and lenses were removed. Eyecups were washed in 0.1 M phosphate buffer. For the frozen sections, the tissues were incubated in 30% sucrose overnight at 4°C. The tissues were embedded in optimal cutting temperature (OCT; Sakura Finetek, Torrance, CA) compound and then frozen at −70°C. Retinal sections were cut to a thickness of 10 μm at −20°C, mounted on glass slides, and stored at −20°C. Endogenous peroxidases were inhibited with 0.3% hydrogen peroxide. Sections were blocked in 2% horse serum and incubated overnight in anti-p-CREB (1:1000 in PBS; New England Biolabs, Beverly, MA) that recognized phosphorylation of serine-133 as a primary antibody. After overnight incubation with the primary antibody, the sections were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG) for an hour, and subsequently streptavidin-conjugated peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated for 30 minutes. The immunoreactivity was detected using a 3,3′-diaminobenzidine detection system (Roche Molecular Biochemicals, Mannheim, Germany) and was observed under a light microscope equipped with a filter (DIC; Olympus, Tokyo, Japan).

Western Blot Analysis

After application of GM1 to the retina with the axotomized nerve, the retina was isolated and stored at −70°C until further use. The retina was incubated in 10 mL of lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin) and then homogenized and incubated on ice for 30 minutes. After centrifugation at 13,000 rpm for 15 minutes, the supernatant was collected to establish total protein values. The amount of protein was determined using a bicinchoninic acid (BCA) protein assay kit (Sigma, St. Louis, MO).

Thirty micrograms of protein from each sample was loaded on SDS-polyacrylamide gels for electrophoresis. After the gel was loaded, the proteins were transferred to a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech, Piscataway, NJ) at 300 mA for 1 hour. Western blot analysis was performed after the membrane was blocked with 5% skim milk. The membrane was incubated with polyclonal rabbit anti-CREB (1:1000) or anti-p-CREB antibodies (1:1000) for 2 hours and washed three times in PBST (PBS containing 0.1% Tween 20) buffer. The membrane was then incubated with horseradish peroxidase (HRP)–conjugated anti-rabbit IgG. After three 10-minute washes with PBST buffer, HRP activity was visualized by applying chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) followed by exposure of the membrane to x-ray film.

The exposed films were analyzed by computer (Image Master VDS, ver. 2.0; Pharmacia Biotech Inc., San Francisco, CA). The density was shown by the ratio of phosphorylated proteins to unphosphorylated proteins. The experiments were performed three times, and the values were expressed as the mean ± SEM.

Retrograde Staining

For quantification of RGCs, the cells were labeled by using a retrograde method by application of 1% fast blue (Sigma) in a sponge (Weck-cel; Edward Weck Inc., Research Triangle Park, NC), after 1 mm was cut from the axotomized nerve end. Retrograde staining was performed 12 days after axotomy, and 48 hours later, the retinas were prepared and fixed in 4% paraformaldehyde and flatmounted on glass slides. Labeled retinal ganglion cells were observed under a fluorescence microscope. Viable cells (i.e., labeled RGCs) were counted in five areas of 1 × 1 mm per retina. Five retinas were used for each group of experiments. For statistical analysis, the counts in each group were taken from three independent experiments, and the groups were compared by unpaired Student's *t*-test. Significance was set at $P < 0.05$, with data reported as the mean ± SEM.

Results

MAPK Activation by GM1

Previous studies have shown that neurotrophins activate MAPK as a Trk family downstream signaling pathway and that

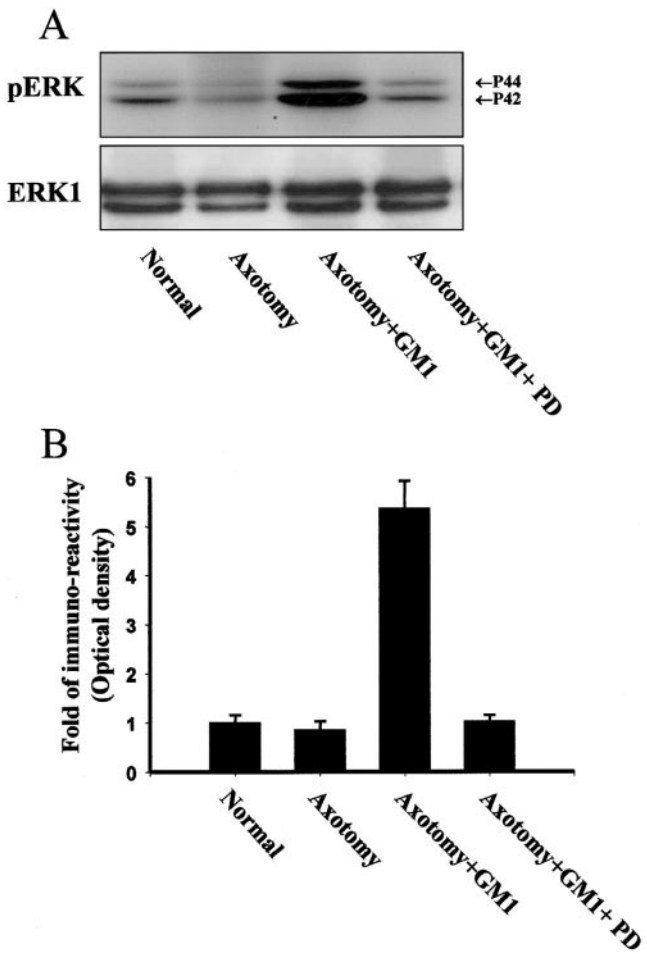

Figure 1. (**A**) ERK phosphorylation 3 days after axotomy. ERK phosphorylation was reduced in the retina with axotomized nerve. However, GM1 treatment increased phosphorylation of ERK, whereas the amount of ERK protein was the same in each lane. The phosphorylation of ERK was inhibited by the MAP kinase inhibitor PD98059. *Top*: p44; *bottom*: p42 ERK. (**B**) Densitometric analysis of ERK shows mean results in three separate experiments (mean ± SEM). The phosphorylation status of ERK was shown by the ratio of phosphorylated proteins to unphosphorylated proteins.

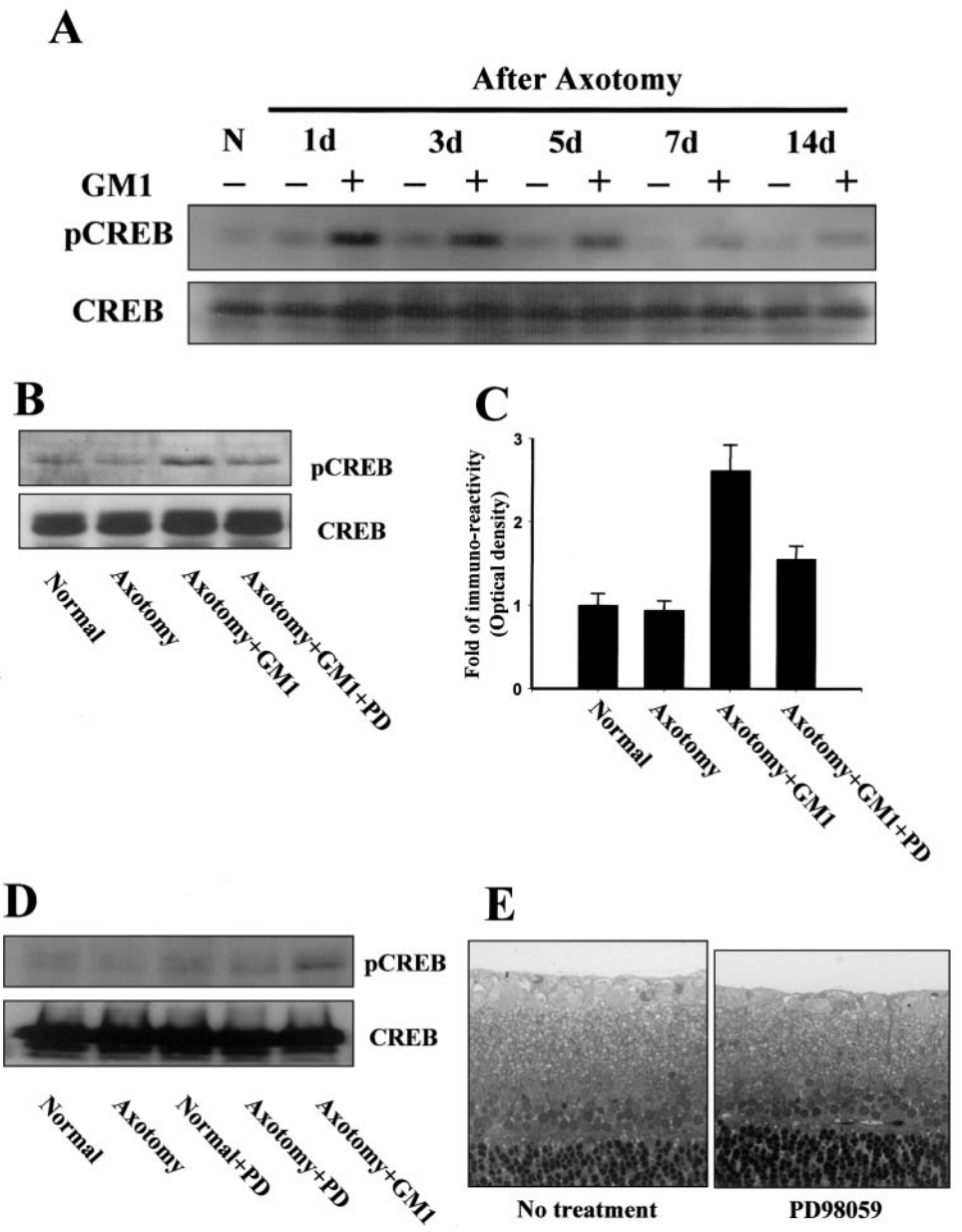

FIGURE 2. (**A**) Western blot analysis of p-CREB in retina with axotomized nerve at various time points after GM1 treatment. Phosphorylation of CREB was observed from 1 day after GM1 treatment in the retina with axotomized nerve, whereas the amount of CREB was not changed by GM1. (**B**) Western blot analysis for phosphorylation of CREB. Retinas were isolated and incubated with cell lysis buffer containing protease and phosphatase inhibitors. The cell lysates were collected and subjected to Western blot analysis. The phosphorylation of CREB was identified with an antibody that recognizes p-CREB. CREB phosphorylation was reduced in the retina with axotomized nerve, but was increased by treatment with GM1, with the same amount of CREB protein in each lane. The MAP kinase inhibitor, PD98059, inhibited the phosphorylation of CREB. (**C**) Densitometric analysis of CREB shows mean results of three separate experiments (mean ± SEM). The phosphorylation status of CREB was determined by the ratio of phosphorylated proteins to unphosphorylated proteins. (**D**) Western blot analysis of p-CREB for the effect of PD98059, 3 days after intravitreous injection of GM1, with or without PD98059. (**E**) Histologic analysis of retina, with and without treatment with 50 μM PD98059.

both neurotrophic factors and GM1 promote neuronal cell survival through phosphorylation of the Trk family.[30] Even though it has been shown that GM1 has a neurotrophic-factor–like effect,[16] it is not known whether GM1 uses the same signal-transduction pathway as neurotrophins do. To examine whether GM1 activates MAPK in the retina with axotomized nerve, we conducted a Western blot analysis. Extracellular signal-regulated protein kinase (ERK) is known to be one of the substrates of MAPK and is phosphorylated when MAPK is activated.[39] Phosphorylation of ERK was almost negligible in control and in the retina with axotomized nerve. However, phosphorylated (p)-ERK was enhanced with the injection of GM1 into vitreous body (Fig. 1A), whereas the amount of ERK in each experiment was the same (Fig. 1B). Next, to determine whether ERK phosphorylation is due to the activation of MAPK, we used PD98059, a specific inhibitor of MAPK. ERK phosphorylation was significantly reduced by treatment with PD98059. The result suggests that GM1 activates the MAPK signaling pathway.

CREB Activation by GM1

There are numerous reports showing that CREB is involved in the survival mechanism of many cell types, including neurons.[23] The activity of the transcription factor CREB is enhanced when Ser133 is phosphorylated by various kinases, such as protein kinase A (PKA), CaM kinase, and MAPK[40] Thus, we conducted a Western blot analysis (Fig. 2) to examine whether the activated MAPK affects the phosphorylation status of CREB. The results (Fig. 2A) show that GM1 enhanced the phosphorylation of CREB from 1 day after GM1 treatment, and persisted until 5 days after treatment. Subsequently, phosphorylation of CREB was greatly reduced at 7 days after GM1 treatment. Although there are many reports showing that MAPK can phosphorylate CREB,[40,41] it has not been reported that GM1 uses the same signaling pathway in the retina with axotomized nerve. To investigate this question, we applied PD98059, a specific inhibitor of MAPK, to examine whether this inhibitor could affect the phosphorylation of CREB. As shown in Figures 2B and 3, PD98059 blocked phosphorylation

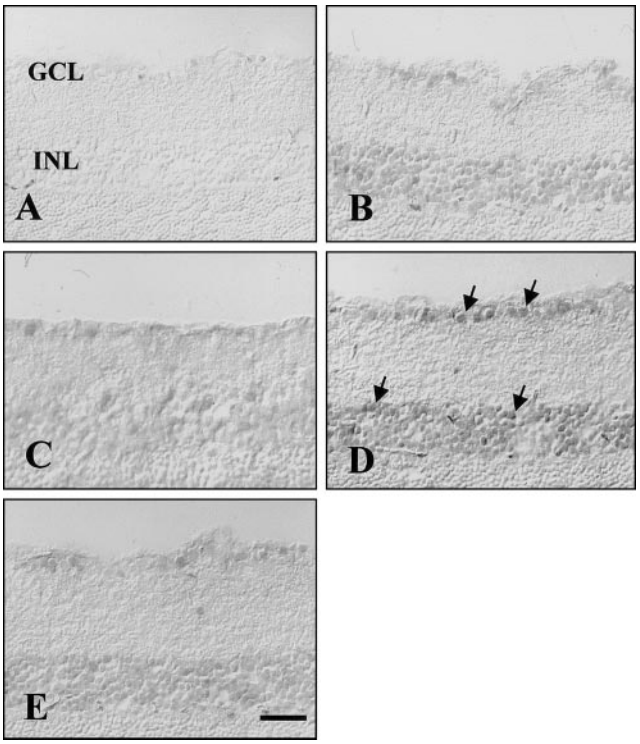

FIGURE 3. Immunohistochemical analysis for p-CREB in the retina with axotomized nerve, with or without GM1-treatment. (**A**) Negative control which omitted only the primary antibody (anti-pCREB antibody). Phosphorylated CREB was not observed in uninjured (normal) retina (**B**), retina with axotomized nerve (**C**), or retina with axotomized nerve treated with both GM1 and PD98059 (**E**). However, p-CREB was observed in the GCL and INL (*arrows*) of the GM1-treated retina with axotomized nerve (**D**). Bar, 25 μm.

of CREB in the retina with axotomized nerve treated with GM1. In contrast, PD98059 alone did not affect the phosphorylation of CREB (Fig. 2D). In addition, histologic examination (Fig. 2E) and retrograde staining (data not shown) showed that PD98059 alone did not affect the viability of the retinal cells. The results imply that GM1 enhances phosphorylation of CREB through the MAPK signaling pathway.

Immunohistochemical Study of p-CREB in Retina with Axotomized Nerve

To detect localization of activated CREB, we performed this experiment in GM1-treated retina. The immunohistochemistry result showed that p-CREB was observed mainly in the ganglion cell layer (GCL) and inner nuclear layer (INL) of GM1-treated retina (Fig. 3). In nontreated retina, p-CREB was not detected in the GCL and INL. The p-CREB in GM1-treated retina was reduced by PD98059 treatment. The results were in agreement with those of Western blot analysis.

Effect of Activation of MAPK by GM1 on Survival of RGCs

Even though GM1 appeared to activate the MAPK signaling pathway, it is not clear whether the activation of MAPK by GM1 is directly related to the survival signal. To examine whether activation of MAPK enhances the survival of retinal cells, we injected GM1, with or without PD98059. We quantified the surviving RGCs with retrograde staining, which specifically detects surviving RGCs among the neurons in the GCL.[42] The surviving RGCs had a round shape made visible by FB labeling (Fig. 4). Twice as many cells (1326 ± 48/mm^2)

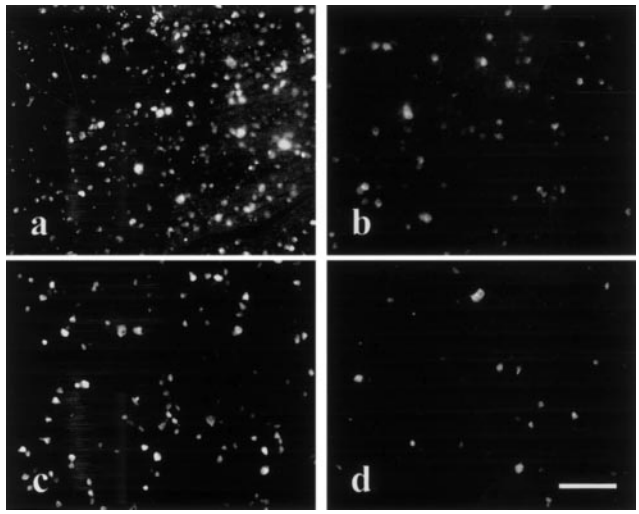

FIGURE 4. Retrograde staining of RGCs in retina. The retina was labeled by fast blue and flatmounts were observed under a fluorescence microscope. (**A**) Normal retina (**B**) vehicle-treated retina, (**C**) GM1-treated retina with axotomized nerve, and (**D**) GM1- and PD98059-treated retina with axotomized nerve. Bar, 100 μm.

survived in GM1-treated retina with axotomized nerve than in retina with axotomized nerve only or PD98059-treated retina with axotomized nerve. The result shows that application of PD98059 reduced the neuroprotective effect of GM1 (Table 1). This result suggests that activation of MAPK plays a role in the neuroprotective activity of GM1.

DISCUSSION

In the present study, GM1 protected retinal ganglion cells after optic nerve injury through enhanced CREB phosphorylation by MAPK. These results suggest that GM1 promotes the survival of injured neuronal cells using mechanisms similar to those generally used by neurotrophic factors. Deprivation of neurotrophic factors is a well-documented cause of neuronal death, both in vivo and in vitro.[43–45] To clarify the mechanism of the neuroprotective effect of GM1, we chose an in vivo model of apoptosis, axotomy.

The survival mechanism of neurotrophic factors has been investigated by many others.[4,25,45,46] Among the known signaling pathways of cell survival, MAPK and PI 3-K seem to be the key activators of many cellular responses that determine cell fate.[25,47] CREB, a point of convergence for MAPK and PI3-K, determines the survival or plasticity of neuronal cells, depending on the stimuli.[23–25,45,47,48] Even though the survival mechanism controlled by phosphorylated CREB in neuronal cells is not clear, many recent reports support the idea that the sur-

TABLE 1. Number of Fast-Blue–Labeled Retinal Ganglion Cells

	n	Percentage
Normal retina	3042 ± 156	100
Axotomy	752 ± 43	24.72
Axotomy+GM1	1326 ± 48	43.59
Axotomy+GM1+PD98059	630 ± 31	20.71

Data are mean number of cells per square millimeter ± SEM. Quantification of fast-blue–labeled (RGCs) 14 days after axotomy. The labeled cells were counted in five regions (1 mm^2) in each retina, and five retinas were used for each experiment.

vival effect is mediated by the regulation of downstream target genes, including *BCL-2*, *BDNF*, and *NGF*.[45,49]

GM1 research has focused on clinical therapy for rescuing neurons, because GM1 is both nontoxic, as a natural component of cellular material, and less costly, compared with the neurotrophic factors. GM1 is known as an activator of the Trk family of receptors, which enhances the dimerization or autophosphorylation of the receptors.[15,20] However, it has not been determined whether CREB plays a role in GM1-mediated neuronal survival. In previous studies, GM1 application activated MAPK and stimulated proliferation of glioma cells, which are normally quiescent.[21] In contrasting results, Ryu et al.[12] showed that GM1 attenuates the cell death caused by serum deprivation by stimulation of PI 3-K, but not the MAPK pathway, in cultured cortical neurons.[12] In addition, GM1 had no effect on the insults of excitotoxins (e.g., *N*-methyl-D-aspartate [NMDA] or kinate) on the cortical neurons. However, GM1 had a partial protective effect on ischemia–reperfusion injury in the rat retina.[12,50]

Our results suggest that p-CREB may be involved in the survival effect of GM1. However, we have not tested whether impaired CREB phosphorylation is directly related to cell death, because our experimental observations were limited to the in vivo test.

Our results showed that GM1 attenuated cell death in the axotomized GCL by approximately 50%, rather than blocking cell death, which is similar to the effect of other neurotrophins. Although GM1 promoted the phosphorylation of CREB, similar to other neurotrophins, the results suggest that GM1 requires an additional survival mechanism to accomplish complete neuronal protection. The combined action of various neurotrophins may have the ability to activate the complete set of survival mechanisms or have a greater effect on the duration or strength of the activation of signaling molecules. Even though GM1 seems to stimulate only a subset of survival mechanisms; alternatively, the strength of the stimulus may be weaker than that of neurotrophins, our results suggest that GM1 could be used as a neuroprotective agent in conjunction with other neurotrophins.

We have presented evidence that GM1 promotes the survival of RGCs through MAPK activation, which leads to phosphorylation of CREB. This study supports the hypothesis that the activation of MAPK and the phosphorylation of CREB play an important role in the action of neuroprotective agents.

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References

1. Raff MC, Barres BA, Burne JF, et al. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science.* 1993;262:695–700.
2. Yavin E, Hama T, Gil S, Guroff G. Nerve growth factor and gangliosides stimulate the release of glycoproteins from PC12 pheochromocytoma cells. *J Neurochem.* 1986;46:794–803.
3. Shen KF, Crain SM. Nerve growth factor rapidly prolongs the action potential of mature sensory ganglion neurons in culture, and this effect requires activation of Gs-coupled excitatory kappa-opioid receptors on these cells. *J Neurosci.* 1994;14:5570–5579.
4. Skaper SD, Negro A, Facci L, Dal Toso R. Brain-derived neurotrophic factor selectively rescues mesencephalic dopaminergic neurons from 2,4,5-trihydroxyphenylalanine-induced injury. *J Neurosci Res.* 1993;34:478–487.
5. Cunha GM, Moraes RA, Moraes GA, et al. Nerve growth factor, ganglioside and vitamin E reverse glutamate cytotoxicity in hippocampal cells. *Eur J Pharmacol.* 1999;367:107–112.
6. Ferrari G, Greene LA. Promotion of neuronal survival by GM1 ganglioside: phenomenology and mechanism of action. *Ann NY Acad Sci.* 1998;845:263–273.
7. Hicks D, Heidinger V, Mohand-Said S, Sahel J, Dreyfus H. Growth factors and gangliosides as neuroprotective agents in excitotoxicity and ischemia. *Gen Pharmacol.* 1998;30:265–273.
8. Ledeen RW. Biology of gangliosides: neuritogenic and neuronotrophic properties. *J Neurosci Res.* 1984;12:147–159.
9. Dreyfus H, Sahel J, Heidinger V, et al. Gangliosides and neurotrophic growth factors in the retina: molecular interactions and applications as neuroprotective agents. *Ann NY Acad Sci.* 1998;845:240–252.
10. Skaper SD, Facci L, Milani D, Leon A. Monosialoganglioside GM1 protects against anoxia-induced neuronal death in vitro. *Exp Neurol.* 1989;106:297–305.
11. Heidinger V, Hicks D, Sahel J, Dreyfus H. Peptide growth factors but not ganglioside protect against excitotoxicity in rat retinal neurons in vitro. *Brain Res.* 1997;767:279–288.
12. Ryu BR, Choi DW, Hartley DM, et al. Attenuation of cortical neuronal apoptosis by gangliosides. *J Pharmacol Exp Ther.* 1999;290:811–816.
13. Ferrari G, Greene LA. Prevention of neuronal apoptotic death by neurotrophic agents and ganglioside GM1: insights and speculations regarding a common mechanism. *Perspect Dev Neurobiol.* 1996;3:93–100.
14. Cavallini L, Venerando R, Miotto G, Alexandre A. Ganglioside GM1 protection from apoptosis of rat heart fibroblasts. *Arch Biochem Biophys.* 1999;370:156–162.
15. Ferrari G, Anderson BL, Stephens RM, Kaplan DR, Greene LA. Prevention of apoptotic neuronal death by GM1 ganglioside; involvement of Trk neurotrophin receptors. *J Biol Chem.* 1995;270:3074–3080.
16. Rabin SJ, Mocchetti I. GM1 ganglioside activates the high-affinity nerve growth factor receptor trkA. *J Neurochem.* 1995;65:347–354.
17. Fadda E, Negro A, Facci L, Skaper SD. Ganglioside GM1 cooperates with brain-derived neurotrophic factor to protect dopaminergic neurons from 6-hydroxydopamine-induced degeneration. *Neurosci Lett.* 1993;159:147–150.
18. Ledeen RW, Wu G, Cannella MS, Oderfeld-Nowak B, Cuello AC. Gangliosides as neurotrophic agents: studies on the mechanism of action. *Acta Neurobiol Exp* (*Warsz*). 1990;50:439–449.
19. Farooqui T, Franklin T, Pearl DK, Yates AJ. Ganglioside GM1 enhances induction by nerve growth factor of a putative dimer of TrkA. *J Neurochem.* 1997;68:2348–2355.
20. Mutoh T, Tokuda A, Miyadai T, Hamaguchi M, Fujiki N. Ganglioside GM1 binds to the Trk protein and regulates receptor function. *Proc Natl Acad Sci USA.* 1995;92:5087–5091.
21. Van Brocklyn JR, Vandenheede JR, Fertel R, Yates AJ, Rampersaud AA. Ganglioside GM1 activates the mitogen-activated protein kinase Erk2 and p70 S6 kinase in U-1242 MG human glioma cells. *J Neurochem.* 1997;69:116–125.
22. Mutoh T, Tokuda A, Inokuchi J, Kuriyama M. Glucosylceramide synthase inhibitor inhibits the action of nerve growth factor in PC12 cells. *J Biol Chem.* 1998;273:26001–26007.
23. Walton MR, Dragunow I. Is CREB a key to neuronal survival? *Trends Neurosci.* 2000;23:48–53.
24. Walton M, Woodgate AM, Muravlev A, et al. CREB phosphorylation promotes nerve cell survival. *J Neurochem.* 1999;73:1836–1842.
25. Bonni A, Brunet A, West AE, et al. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms [see comments]. *Science.* 1999;286:1358–1362.
26. Robinson GA. Changes in the expression of transcription factors ATF-2 and Fra-2 after axotomy and during regeneration in rat retinal ganglion cells. *Brain Res Mol Brain Res.* 1996;41:57–64.
27. Huxlin KR, Bennett MR. NADPH diaphorase expression in the rat retina after axotomy: a supportive role for nitric oxide. *Eur J Neurosci.* 1995;7:2226–2239.
28. Castagne V, Clarke PG. Neuroprotective effects of a new glutathione peroxidase mimetic on neurons of the chick embryo's retina. *J Neurosci Res.* 2000;59:497–503.

29. Castagne V, Clarke PG. Axotomy-induced retinal ganglion cell death in development: its time-course and its diminution by antioxidants. *Proc R Soc Lond B Biol Sci.* 1996;263:1193–1197.
30. Klocker N, Cellerino A, Bahr M. Free radical scavenging and inhibition of nitric oxide synthase potentiates the neurotrophic effects of brain-derived neurotrophic factor on axotomized retinal ganglion cells In vivo. *J Neurosci.* 1998;18:1038–1046.
31. Castagne V, Barneoud P, Clarke PG. Protection of axotomized ganglion cells by salicylic acid. *Brain Res.* 1999;840:162–166.
32. Cohen A, Bray GM, Aguayo AJ. Neurotrophin-4/5 (NT-4/5) increases adult rat retinal ganglion cell survival and neurite outgrowth in vitro. *J Neurobiol.* 1994;25:953–959.
33. Di Polo A, Aigner LJ, Dunn RJ, Bray GM, Aguayo AJ. Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Muller cells temporarily rescues injured retinal ganglion cells. *Proc Natl Acad Sci USA.* 1998;95:3978–3983.
34. Frade JM, Bovolenta P, Rodriguez-Tebar A. Neurotrophins and other growth factors in the generation of retinal neurons. *Microsc Res Tech.* 1999;45:243–251.
35. Ikeda K, Tanihara H, Honda Y, et al. BDNF attenuates retinal cell death caused by chemically induced hypoxia in rats. *Invest Ophthalmol Vis Sci.* 1999;40:2130–2140.
36. Peinado-Ramon P, Salvador M, Villegas-Perez MP, Vidal-Sanz M. Effects of axotomy and intraocular administration of NT-4, NT-3, and brain-derived neurotrophic factor on the survival of adult rat retinal ganglion cells: a quantitative in vivo study. *Invest Ophthalmol Vis Sci.* 1996;37:489–500.
37. Yan Q, Wang J, Matheson CR, Urich JL. Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of axotomized retinal ganglion cells in adult rats: comparison to and combination with brain-derived neurotrophic factor (BDNF). *J Neurobiol.* 1999; 38:382–390.
38. Ferrari G, Batistatou A, Greene LA. Gangliosides rescue neuronal cells from death after trophic factor deprivation. *J Neurosci.* 1993; 13:1879–1887.
39. Rampersaud AA, Van Brocklyn JR, Yates AJ. GM1 activates the MAP kinase cascade through a novel wortmannin-sensitive step upstream from c-Raf. *Ann NY Acad Sci.* 1998;845:424.
40. Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem.* 1999;68:821–861.
41. Schmid RS, Graff RD, Schaller MD, et al. NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells. *J Neurobiol.* 1999;38:542–558.
42. Chaouat G, Menu E, Kinsky R, Brezin C. Immunologically mediated abortions: one or several pathways? *Res Immunol.* 1990;141:188–195.
43. Watanabe M, Sawai H, Fukuda Y. Survival of axotomized retinal ganglion cells in adult mammals. *Clin Neurosci.* 1997;4:233–239.
44. Mey J, Thanos S. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. *Brain Res.* 1993;602:304–317.
45. Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science.* 1999;286:2358–2361.
46. D'Mello SR, Galli C, Ciotti T, Calissano P. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc Natl Acad Sci USA.* 1993;90:10989–10993.
47. Du K, Montminy M. CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem.* 1998;273:32377–32379.
48. Finkbeiner S. CREB couples neurotrophin signals to survival messages. *Neuron.* 2000;25:11–14.
49. Pugazhenthi S, Nesterova A, Sable C, et al. Akt/Protein kinase B up-regulates bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem.* 2000;275:10761–10766.
50. Weber M, Mohand-Said S, Hicks D, Dreyfus H, Sahel JA. Monosialoganglioside GM1 reduces ischemia–perfusion-induced injury in the rat retina. *Invest Ophthalmol Vis Sci.* 1996;37:267–273.