Brain-Derived Neurotrophic Factor Prevents Axotomized Retinal Ganglion Cell Death through MAPK and PI3K Signaling Pathways

Toru Nakazawa,[1,2] *Makoto Tamai,*[2] *and Nozomu Mori*[1,3]

Purpose. Brain-derived neurotrophic factor (BDNF) has a potential neuroprotective effect on axotomized retinal ganglion cells (RGCs); however, the mechanism, in regard to intracellular signaling, of BDNF-induced neuroprotection of RGCs is largely unknown. Intracellular signaling was investigated, by using axotomized RGCs and the relative contribution of the two major downstream signaling routes of TrkB determined—that is, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)-Akt routes, mediated by BDNF.

Methods. Neuroprotective effects of BDNF were determined by quantifying the surviving RGCs after axotomy, by retrograde labeling. The MAPK and Akt levels were determined by Western blot analysis and activity assays. Quantification of the relative contribution of the two signaling pathways was performed by use of specific inhibitors for MAPK and PI3K (i.e., U0126 and LY294002, respectively).

Results. Intravitreous administration of BDNF had the most profound neuroprotective effects on axotomized RGCs among the neurotrophins. Burst phosphorylation of MAPK and Akt was induced by BDNF within 1 hour and was sustained over 2 weeks in the whole retina. Immunohistochemistry revealed that phosphorylated MAPK was detected in the RGCs and retinal Müller cells, and Akt was in the RGCs. BDNF-induced phosphorylation of MAPK and Akt was suppressed by their specific inhibitors. Moreover, administration of U0126 and LY294002 decreased significantly, but only partially, the neuroprotective effect of BDNF on the axotomized RGCs.

Conclusions. BDNF-mediated signaling involves activation of both MAPK and Akt on the axotomized adult rat retina, and the collaboration of both MAPK and PI3K-Akt pathways seems to be necessary in neuroprotective signaling in axotomized RGCs. (*Invest Ophthalmol Vis Sci.* 2002;43:3319–3326)

From the [1]Department of Molecular Genetics, National Institute for Longevity Sciences (NILS), Aichi Japan; the [2]Department of Ophthalmology, Tohoku University School of Medicine, Miyagi, Japan; and the [3]Program of Protecting the Brain, Core Research for Evolutional Science and Technology (CREST), Science and Technology Corporation of Japan (JST), Aichi, Japan.

Supported by the Special Coordination Funds for Promoting Science and Technology, the Strategic Promotion System for Brain Science of the Science and Technology Agency of Japan (NM) and (MT); Comprehensive Research on Aging and Health from the Ministry of Health, Labor and Welfare; the CREST program of the Science and Technology Corporation of Japan (NM); and Grant 10307041 from the Developmental Scientific Research of Ministry of Education, Science and Culture of Japan (MT).

Submitted for publication April 4, 2002; accepted May 20, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Makoto Tamai, Department of Ophthalmology, Tohoku University School of Medicine, Sendai, Miyagi 980-8574, Japan; mtamai@oph.med.tohoku.ac.jp.

Neurotrophins, the so-called nerve growth factor (NGF) family members, have multiple functions in both developing and mature neurons (for review, see Ref. 1). Among the neurotrophins, the brain-derived neurotrophic factor (BDNF) has been well characterized regarding its effects on regeneration, synaptic modulation, and neuroprotection.[2] In the visual system, the neuroprotective effect of BDNF was demonstrated in the various retinal injury models, including retinal ganglion cell (RGC) axotomy,[3–5] glutamate neurotoxicity,[6] ischemia–reperfusion injury,[7] and photograph-induced damage.[8] The neuroprotective effects of BDNF are potent, but transient, as demonstrated by the fact that repeated intravitreous injection of BDNF proteins or even prolonged transfection with BDNF-expressing adenoviral vectors still had only a limited neuroprotective effect against RGC axotomy.[9] The reason for this transient effect could be the downregulation or internalization of trkB, which is the receptor for BDNF, or the induced expression of truncated trkB isoforms.[10,11] For an understanding of the mechanisms of BDNF-mediated neuroprotection, it is important to explore the signal transduction mechanism elicited by trkB activation in RGCs.

On activation by BDNF, trkB initiates intracellular signaling through two major signal output regions: Shc and PLCγ binding sites.[12] Among these trkB signal output regions, the Shc binding site plays major roles in neuronal survival and axonal outgrowth.[12] Data in our prior studies have shown that N-Shc/ShcC, rather than ShcA, is the key adapter protein toward Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K) in BDNF/TrkB-induced intracellular signaling in the central nervous system.[13–16] MAPK and PI3K pathways have been well characterized in various neuronal subsets.[17–19] The roles of these pathways, however, are distinct in each neuronal subtype. For example, in PC12 cells, prolonged activation of MAPK was sufficient for cell survival after withdrawal of the NGF.[20] The MAPK signaling pathway promotes cell survival through a dual mechanism comprising the inactivation of proapoptotic protein and the increased transcription of prosurvival genes, such as cAMP-responsive element-binding (CREB) protein.[21] However, recent evidence indicates that the PI3K/Akt pathway is more relevant to cell survival than the MAPK pathway in cerebellar granular neurons[22] and spinal motor neurons.[17]

Even in the same type of neuron, different signal transduction pathways could be used, depending on the type of neuronal damage or injury. For example, in primary cultures of cortical neuron, the MAPK pathway plays a major role in BDNF-mediated neuroprotection against camptothecin; however, the PI3K pathway is dominant in BDNF-mediated neuroprotection against the withdrawal of serum.[19] Thus, the downstream signaling mechanisms of BDNF-trkB–mediated neuroprotection are still controversial. In the retina, the MAPK pathway was demonstrated to play pivotal roles in the extension of RGC axons,[23] as well as in the promotion of survival of RGCs in postnatal rats in vitro and in vivo.[10,24] In the adult rat retina, inhibition of caspase-3 also rescued axotomized RGCs from secondary cell death in vivo,[25,26] and the inhibition of the caspase activity was mediated, at least in part, by the activation

of Akt induced by insulin-like growth factor (IGF)-1 and BDNF.[27,28] It is thus still unclear which signaling route(s) downstream of TrkB are used in the process of BDNF-mediated neuroprotection and/or regeneration after RGC axotomy.

The retinotectal system is a useful model for investigating the molecular mechanisms of neuronal apoptosis and survival in vivo during development and in the adult central nervous system (CNS).[29,30] In this system, particularly including the paradigm of RGC axotomy, we determined and compared the relative contribution of the MAPK and PI3K-Akt pathways, which are downstream of ShcC/Sos/Ras, in BDNF-mediated neuroprotective signaling on axotomized RGCs in vivo.

Materials and Methods

Animals

Two hundred eighty-two male Sprague-Dawley (SD) rats (male, weighing 300–340 g; Japan SLC, Hamamatsu, Japan) were prepared and divided into two groups in this study. One hundred fourteen of them were used for morphologic study, including immunohistochemistry and cell counting, and the other 168 for biochemical study, including protein activity and Western blot analysis. All animals were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Surgical and Retrograde Labeling

Transection of the optic nerve (ON) entering the right eye was performed as previously described.[16,30,31] Briefly, adult SD rats were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg body weight). After a skin incision had been made close to the superior orbital rim, the orbita was opened with care taken to leave the vortex vein intact. After resection of the superior extraocular muscles, the optic nerve was exposed by longitudinal incision of the perineurium. Transection was performed approximately 1 mm from the posterior eye pole without damaging the retinal blood supply. Animals with persistent retinal ischemia verified funduscopically were excluded. In a subset of animals assigned to the neuroprotection study, RGCs were retrogradely labeled with gold fluorochrome (Fluorogold; Fluorochrome, Englewood, CO). Labeling was performed by aspirating the cerebrum and placing a small piece of gel foam soaked in 2% aqueous fluorochrome containing 1% dimethyl sulfoxide (DMSO) at the bilateral superior colliculi (SC) 7 days before the transection surgery or by placing a piece of gelfoam soaked in 2% aqueous fluorochrome containing 1% DMSO on the stump of the transected ON.[30]

Counting the Fluorochrome-Labeled RGCs in the Flat-Mounted Retina

Rats were killed by an overdose of pentobarbital at 10 days after ON transection. Retinas were dissected, fixed in 4% paraformaldehyde (PFA) and flatmounted onto glass slides. Cell counting was performed as previously described[30] under a fluorescence microscope (Leica Microsystems, Heidelberg, Germany) using a UV filter set (365/397 nm). RGC densities were determined by counting the tracer-labeled RGCs in 12 distinct areas of 7.29×10^{-2} mm^2 each (three areas per retinal quadrant at ⅙, ⅜, and ⅝ of the retinal radius). The total area of the 12 represented fields was 8.75×10^{-1} mm^2, which was more than the area counted by Kermer et al.[27] by approximately 120%. The density of fluorochrome-labeled RGCs was defined as the average number of cells in the 12 fields counted and was analyzed statistically[30] (see Fig. 1 for details). To reduce bias, cell counting was performed and confirmed by three independent investigators in a masked fashion. Using this counting procedure, the mean counts made in each area by the three investigators were within the mean ± 10%; moreover, the average counts in the 12 fields of 29.16 mm^2 each (four times) were within the mean ± 5% (data not shown).

Drug Administration and Tissue Processing

Recombinant human β-NGF or BDNF (PeproTech House, London, UK) or NT-3 (a gift of Takeda Chemical Industries, Osaka, Japan) was dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). U0126 (Promega, Madison, WI) and LY294002 (Calbiochem) were dissolved in DMSO. Both solutions were further diluted in PBS-BSA. For intravitreous drug injection, animals were anesthetized and 1 μg of neurotrophin or PBS/BSA (3 μL) was injected into the vitreous space within 1 minute by puncturing the eye at the corneal–scleral junction by means of a syringe (Hamilton, Reno, NV) equipped with a 32-gauge needle. In a subset of the animals, 0.15 nmol per eye U0126 and 0.6 nmol per eye LY294002 or PBS-BSA with 5% DMSO was injected into the vitreous space 30 minutes before the ON transection (see Fig. 4C).

Western Blot Analysis

Retinal protein was extracted with lysis buffer (10 mM Tris [pH 7.5]; 1% TritonX-100; 0.5% NP-40; 1 mM EDTA; 150 mM NaCl; 1 μg/mL each of aprotin, leupeptin, antipain, and pepstatin A; 1 mM *p*-amidinophenyl methanesulfonyl fluoride; 10 mM *p*-nitrophenyl phosphate; 5 mM sodium pyrophosphate; 0.2 mM sodium molybdate; 10 mM β-glycerophosphate; 50 μM sodium fluoride; 1 mM sodium orthovanadate) on ice for 30 minutes and was then centrifuged at 15,000 rpm at 4°C for 15 minutes. The protein in the supernatant of the lysate was quantified by use of BCA (Pierce, Rockford, IL). An aliquot of this total protein was mixed with SDS-buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 6% β-mercaptoethanol), boiled for 5 minutes, and processed for SDS-PAGE (20 μg protein for MAPK or 40 μg for Akt). Proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA), preincubated in a blocking buffer (Tris-buffered saline with 0.1% Tween 20 containing 3% BSA) for 1 hour, or incubated first with phosphospecific antibodies (i.e., a rabbit anti-phosphorylated MAPK antibody; dilution 1:5000, Promega) and a rabbit anti-phosphorylated Akt antibody (1:1000; New England Biolabs [NEB], Frankfurt am Main, Germany) overnight at 4°C. Subsequently, the labeled proteins were reacted with an alkaline phosphatase-conjugated anti-rabbit IgG (1:20,000; Promega) followed by visualization by chemiluminescence (CDP-Star; Amersham Biosciences, Buckinghamshire, UK). After this phosphospecific blot had been obtained, the membranes were incubated in stripping buffer (10 mM Tris [pH 8.0], 2% SDS, and 2-mercaptometanol) at 50°C for 30 minutes. These stripped membranes were then washed three times, blocked, and incubated in blocking buffer containing a rabbit anti-MAPK antibody (1:1000; NEB) or a rabbit anti-Akt antibody (1:1000; NEB) overnight at 4°C. Subsequently, they were visualized as just described.

Protein Kinase Activity Assay

Total retinal protein was extracted with lysis buffer as described earlier, quantified by BCA, subdivided into aliquots each containing 10 μg protein, and stored at −80°C until use. The protein kinase activity assay was performed by using the p42/p44 MAP kinase enzyme assay system (Amersham Biosciences), according to the instructions of the manufacturer.

Immunohistochemistry

One hour after intravitreous administration of PBS ($n = 6$) or BDNF ($n = 8$), adult rat retinas were fixed with 4% paraformaldehyde (PFA) at 4°C overnight. They were cryoprotected in 10% to 20% sucrose-PBS and then sectioned (10 μm) including the optic nerve. The sections were mounted onto slides and incubated with blocking buffer (PBS containing 10% goat serum, 0.5% gelatin, 3% BSA, and 0.2% Tween 20) at room temperature (RT) for 1 hour. After three washes in 0.1 M PBS, the sections were incubated overnight at 4°C in a solution of anti-phosphorylated MAPK antiserum (1:250; NEB) or anti-phosphorylated Akt antiserum (1:250; NEB). For the negative control, we used a control solution of anti-rabbit antiserum (X0903; Dako, Osaka, Japan). The sections were then rinsed three times in 0.1M PBS and incubated

with Cy3-conjugated anti-rabbit IgG antibody solution (1:250; Amersham Biosciences) at RT for 60 minutes, followed by three rinses with 0.1 M PBS. Sections were mounted with antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Photomicrographs were taken 1 mm off the center of optic nerve with a confocal laser system (Radiance 2000; BioRad Laboratory, Hercules, CA).

Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by the Scheffé post hoc test for planned comparisons among the various treatments. The significance levels were set at $P < 0.05$ and $P < 0.01$. Data are expressed as the mean ± SD and the probability determined in comparison with the vehicle-treated retina.

Results

Effect of BDNF on Axotomized RGCs In Vivo

Systemic counting of retrogradely labeled RGCs from the SC showed that the total number for the control retina was 2436.48 ± 383.41 cells/mm^2 ($n = 8$), which was close to that previously reported.[32] When the optic nerve was transected at approximately 1 mm from the eyeball, the total number of surviving RGCs decreased to 750.83 ± 44.84/mm^2 ($n = 8$) at 10 days after lesion, significantly lower than that in the control retina ($P < 0.0001$). The reduction in the number of surviving RGCs was prevented by the addition of growth factors. Among the neurotrophins (the NGF family members) tested, BDNF had the most powerful neuroprotective effect on the axotomized RGCs; the remaining RGCs were 703.78 ± 112.38/mm^2, 1840.35 ± 150.28/mm^2, 919.80 ± 55.65/mm^2 for NGF, BDNF, and NT-3, respectively ($n = 8$, Fig. 1B). PBS was administered intravitreously as a surgical control experiment, and the remaining RGCs did not differ significantly in number from those after axotomy alone (823.31 ± 109.73/mm^2; $n = 7$). Therefore, we focused on BDNF to explore the downstream signal transduction for promotion of RGC survival.

Activation of MAPK and PI3K/Akt Signaling Pathways on the Axotomized Retina

To explore BDNF-induced signal transduction on the axotomized retina, we examined the activation of MAPK and Akt, which are, in general, well-characterized trkB-mediated differentiation and survival pathways, respectively. Kinase activity assay and Western blot analysis showed that both MAPK and Akt were activated robustly at 1 hour after administration of BDNF and activation was sustained at least until 3 to 7 days (Figs. 2B, 2E; $n = 4$, respectively). Expression of the unphosphorylated MAPK and Akt proteins remained unchanged significantly in the various experimental groups.

Location of Active MAPK and Akt Induced on the RGCs by BDNF

To clarify the types of target cells of BDNF, we then visualized activated kinases in the cells of rat retina by use of phosphospecific antibodies. In untreated ($n = 6$) and PBS ($n = 6$)-treated retinas, phosphorylated MAPK or Akt was not observed (Figs. 3A, 3B, 3D, 3E). The immunoreactivity indicating phosphorylated MAPK increased in the inner margin of retina, ganglion cell layer (GCL), inner plexiform layer (IPL), and middle layer of the inner nuclear layer (INL) 1 hour after intravitreous injection of BDNF (Fig. 3C; $n = 8$). Phosphorylated Akt was detected in most cells of GCL. In the GCL, there were RGCs and displaced amacrine neurons. We therefore also stained retrogradely labeled fluorochrome-positive RGCs with anti-phosphorylated MAPK or Akt and showed that the activation of these signaling proteins occurred in the RGCs (Fig. 3C, 3F-3, arrows). Phosphorylated MAPK was also detected in the retinal

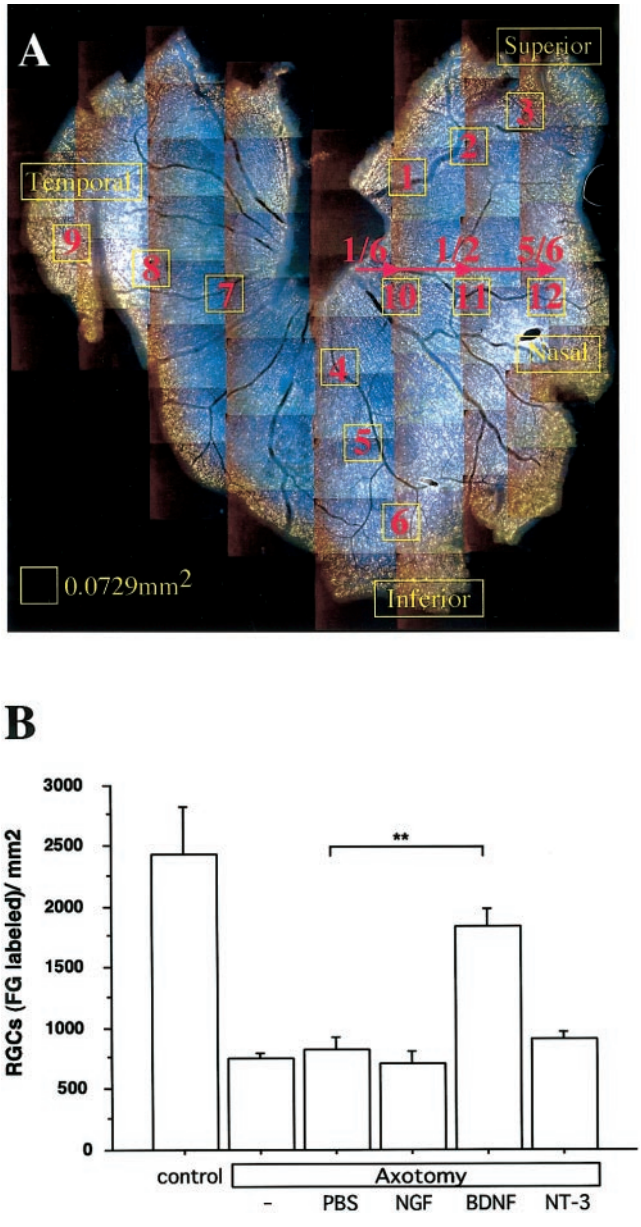

Figure 1. Representative photomicrographs of fluorochrome-labeled flatmount retinas and neuroprotective effects of neurotrophins. (**A**) Representative photomicrograph of flatmount retina and areas in which cells were counted. (**B**) Density (in cells per square millimeter; mean ± SD) of fluorochrome-labeled RGCs from the SC at 10 days after axotomy with concomitant administration of neurotrophin is shown (**$P < 0.01$, ANOVA followed by the Scheffé post hoc test, $n = 7$ or 8). Average RGCs ($n1+\ldots+n12$)/12/0.0729, where n is the number of fluorochrome-labeled RGCs. BDNF had the most powerful neuroprotective effect among the neurotrophins tested.

Müller cells (RMCs), in the inner margin of the retina, and in cells located in the middle layer of the INL a more intense signal than that in the RGCs (Fig. 3C-1, arrowhead).

Effect of Activation of MAPK and Akt on Survival of Axotomized RGCs

In an attempt to differentiate major neuroprotective signal transduction pathways activated by BDNF, we showed the phosphorylation status of MAPK and Akt on the axotomized retina (Fig. 2). We further tested the effects of MAPK and PI3K inhibitors on the BDNF-mediated RGC neuroprotection. In Fig. 4C, we show the time course of the inhibitor experiment. We

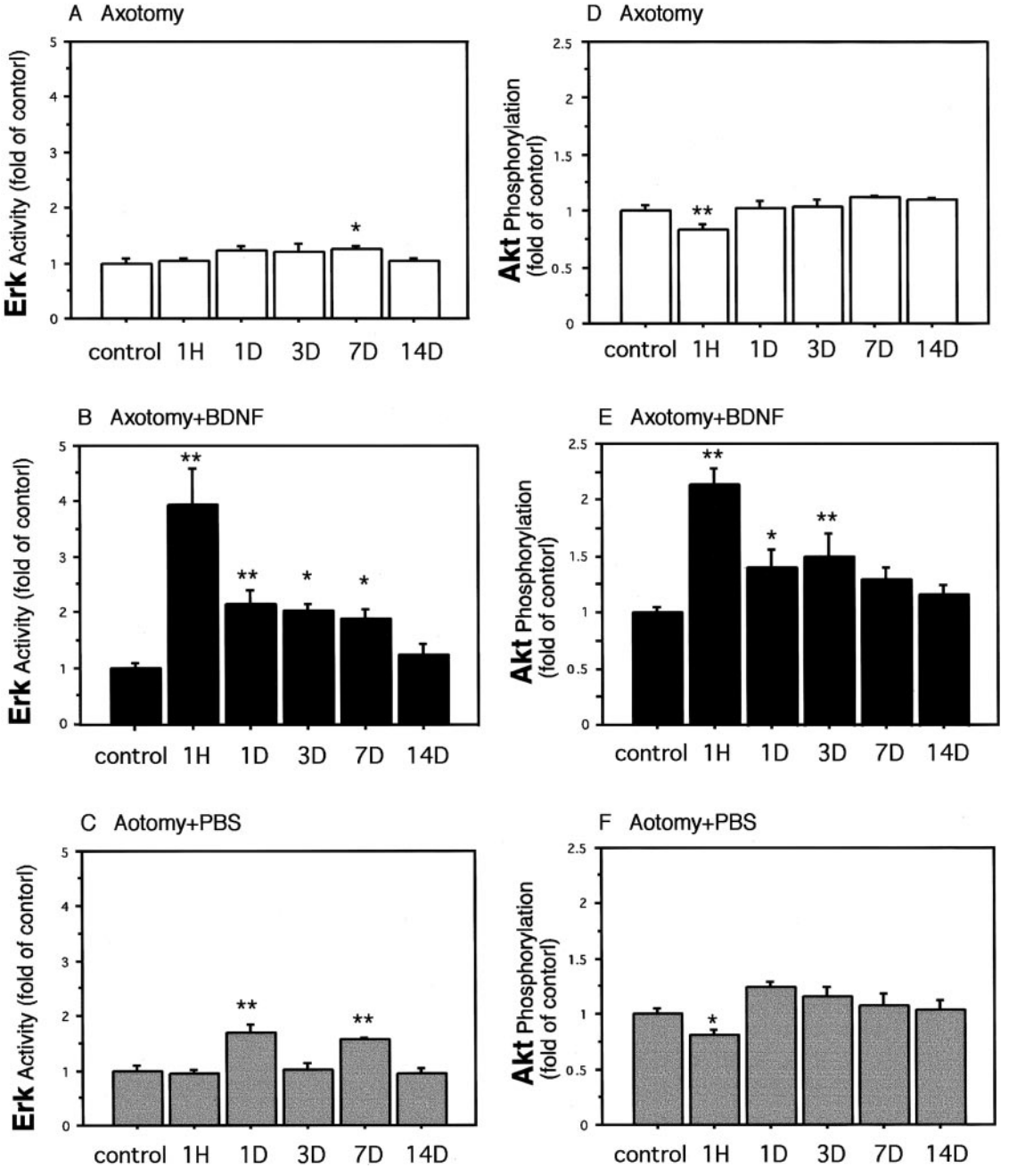

FIGURE 2. Temporal course of activation of MAPK (**A–C**) and Akt (**D–F**) induced by BDNF. Burst activation of MAPK and Akt at 1 hour after axotomy, with simultaneous intravitreous injection of BDNF. This activation was sustained over 3 days ($^{**}P < 0.01$, $^{*}P < 0.05$ ANOVA followed by the Scheffé post hoc test, at each time point, $n = 6$ or 4). H; hours after lesion; D, day after lesion.

injected the MAPK or PI3K inhibitor 30 minutes before administering BDNF. In the preliminary examination, we first coinjected the MAPK inhibitor PD98059 (at 1, 2, 4, and 8 nmol per eye) and BDNF and measured the activity of MAPK after 1 hour. This treatment did not inhibit the activation 1 hour later of MAPK induced by BDNF. However, the injection of PD98059 30 minutes before BDNF inhibited moderately the MAPK activity induced by BDNF. Second, we compared the efficiency of PD98059 and U0126 or LY294002 and wortmannin for inhibition of the respective MAPK or Akt activity. U0126 more strongly suppressed the MAPK activity than PD98059. The high concentration of wortmannin had a suppressive effect on not only Akt but also MAPK activity. Thus, we chose U0126 and LY294002 as the respective MAPK and PI3K-specific inhibitors. Third, we evaluated the concentration of each inhibitor. We injected U0126 (0.15, 1.5, and 15 nmol per eye) or LY294002 (0.06, 0.6, and 6 nmol per eye) 30 minutes before axotomy and administration of BDNF and determined the proper concentration of each inhibitor based on the best inhibition of protein activity (data not shown). As shown in Figures 4A and 4B, specific inhibitors of MAPK and PI3K decreased the BDNF-induced phosphorylation of MAPK (ERK1, 3.61 ± 0.24 [multiples of control levels] to 1.05 ± 0.10; ERK2, 1.65 ± 0.06 to 0.94 ± 0.16, $n = 6$) as well as that of Akt (1.74 ± 0.08 to 1.12 ± 0.05, $n = 6$) to control levels. These results indicate that both MAPK and PI3K-Akt signals are used in the BDNF-dependent signaling in the RGCs.

To distinguish which of the MAPK and PI3K-Akt pathways has a role in promoting the survival of RGCs, we used the same specific inhibitors. In Fig. 5, representative RGC density and

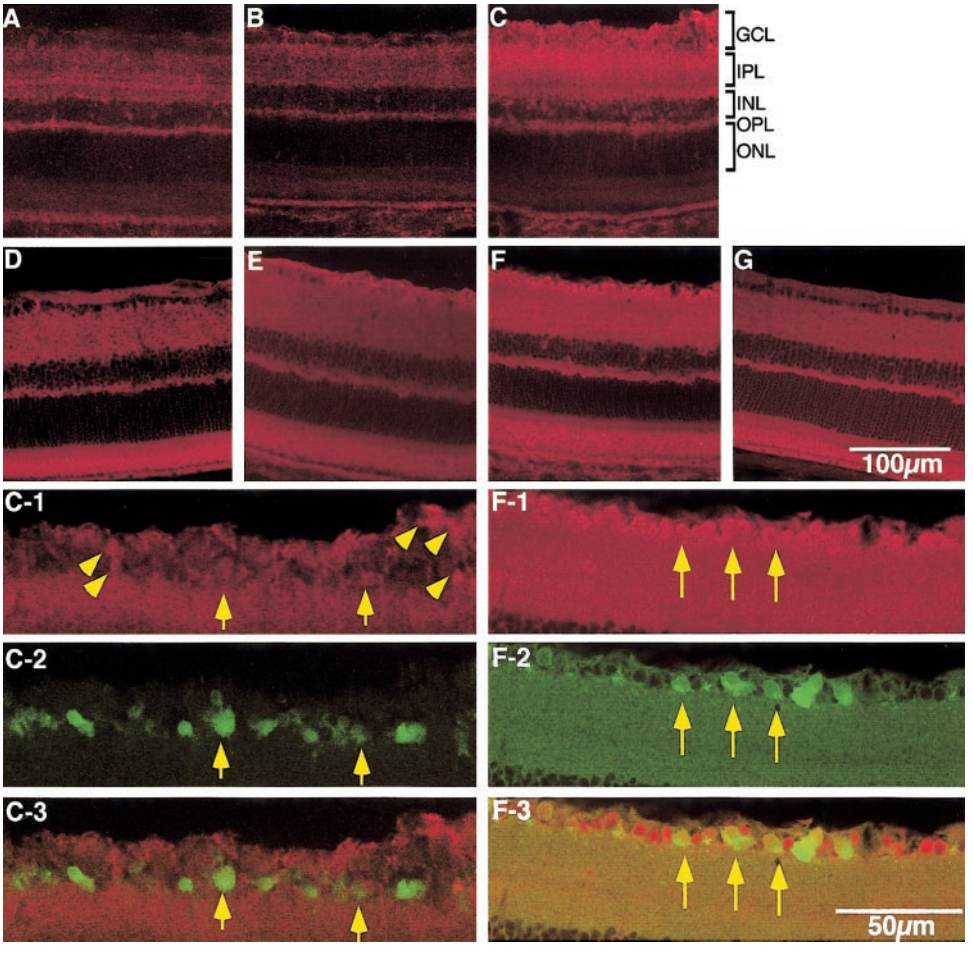

FIGURE 3. Localization of phosphorylated MAPK and Akt in the adult rat retina 1 hour after intravitreous administration of BDNF. Immunohistochemistry of phosphorylated MAPK (**A–C**) and Akt (**D–F**). (**A**, **D**) untreated retina ($n = 6$). One hour after intravitreous administration of (**B**, **E**) PBS ($n = 6$) and (**C**, **F**) BDNF ($n = 8$). (**G**) Negative control. (**C-1–C-3**, **F-1–F-3**) High-magnification images of the GCL of (**C**) and (**F**). (**C**, **F-2**) Fluorochrome-labeled RGCs. (**C**, **F-3**) Merged image. (**C-1–F-3**, *yellow arrows*) RGCs with immunostaining of phosphorylated MAPK and Akt; (**C-1**, *yellow arrowheads*) Müller cells.

morphology are shown at day 10 after lesion, with and without axotomy, together with BDNF and/or kinase inhibitors (i.e., U0126 and LY294002). The results demonstrated that the number of RGCs was reduced by axotomy (compare Fig. 5B with Fig. 5A); however, in BDNF-administered retina, the number of RGCs (1861.8 ± 159.7/mm^2; $n = 11$) was comparable to that of the control unlesioned retina (see Fig. 5D, compared with Fig. 5A). The vehicle control (5% DMSO-PBS) showed essentially the same result as axotomy alone (see Figs. 5B, 5C). Intravitreous injection of kinase inhibitors together with BDNF abolished, at least in part, the neuroprotective effect of BDNF (Fig. 5E: U0126, 1170.30 ± 202.08/mm^2; $n = 11$, $P < 0.0001$; 5F: LY294002, 1178.90 ± 263.20/mm^2; $n = 10$, $P < 0.0001$). Quantification of the number of remaining labeled RGCs from the ON is summarized in Figure 5G. The effects of the drugs were incomplete. Even the combination (965.08 ± 101.03/mm^2; $n = 7$) never reduced the number of remaining RGCs with BDNF to the level of axotomy with the vehicle injection (527.55 ± 67.15/mm^2; $n = 8$). This fact may suggest that the inhibition of kinase activity by the inhibitors does not last as long as the positive effect of BDNF, that BDNF signaling in RGCs further uses other signaling pathways in addition to the MAPK and PI3K-Akt pathways, or that target cells other than RGCs may exist on the axotomized retina.

DISCUSSION

In the present study, we first demonstrated that BDNF had a trophic effect on preventing RGC death after axotomy and further investigated the downstream mechanisms of BDNF/TrkB-mediated signaling on the axotomized RGCs, particularly the activation of MAPK and Akt-mediated signaling routes. The activation of both MAPK and Akt occurred within 1 hour of administration of BDNF and RGC axotomy, but their activation was unexpectedly sustained until 3 to 7 days after lesion. There are possibilities of a differential signaling propagation in RGCs and surrounding cells, which may be sustained over days after axotomy. The burst activation of MAPK was detected in the RGCs and RMCs, suggesting that the signaling response had both a direct effect and secondary effect through cell–cell interaction on the axotomized RGCs. In contrast, the burst activation of Akt was most evident in the RGCs, suggesting that the signaling response had a direct effect on the axotomized RGCs.

Although we demonstrated a significant contribution of activation of both MAPK and Akt after administration of BDNF to the axotomized retina, Klöcker et al.[28] recently reported similar findings that BDNF induces the activation of MAPK and Akt, whereas they had earlier concluded that caspase-3 was a major mediator of the secondary cell death of the axotomized RGCs.[27] A major difference between our system and theirs was the time course of the experiments. They examined the inhibition of activation of MAPK and Akt with specific inhibitor together with BDNF after 4 hours, whereas our results indicate that the activation of both MAPK and Akt peaked after approximately 1 hour of treatment of the axotomized retina with BDNF. In our preliminary examination (not shown) we tested to determine adequate concentrations of the MAPK inhibitor PD98059 to inhibit MAPK by adding the drug simultaneously with BDNF; however, the activation of MAPK was not inhibited, even at high concentrations of PD98059 when the drug was administered together with BDNF. Therefore, we injected these specific inhibitors before the intravitreous injection of BDNF. Because BDNF would elicit an acute response, it

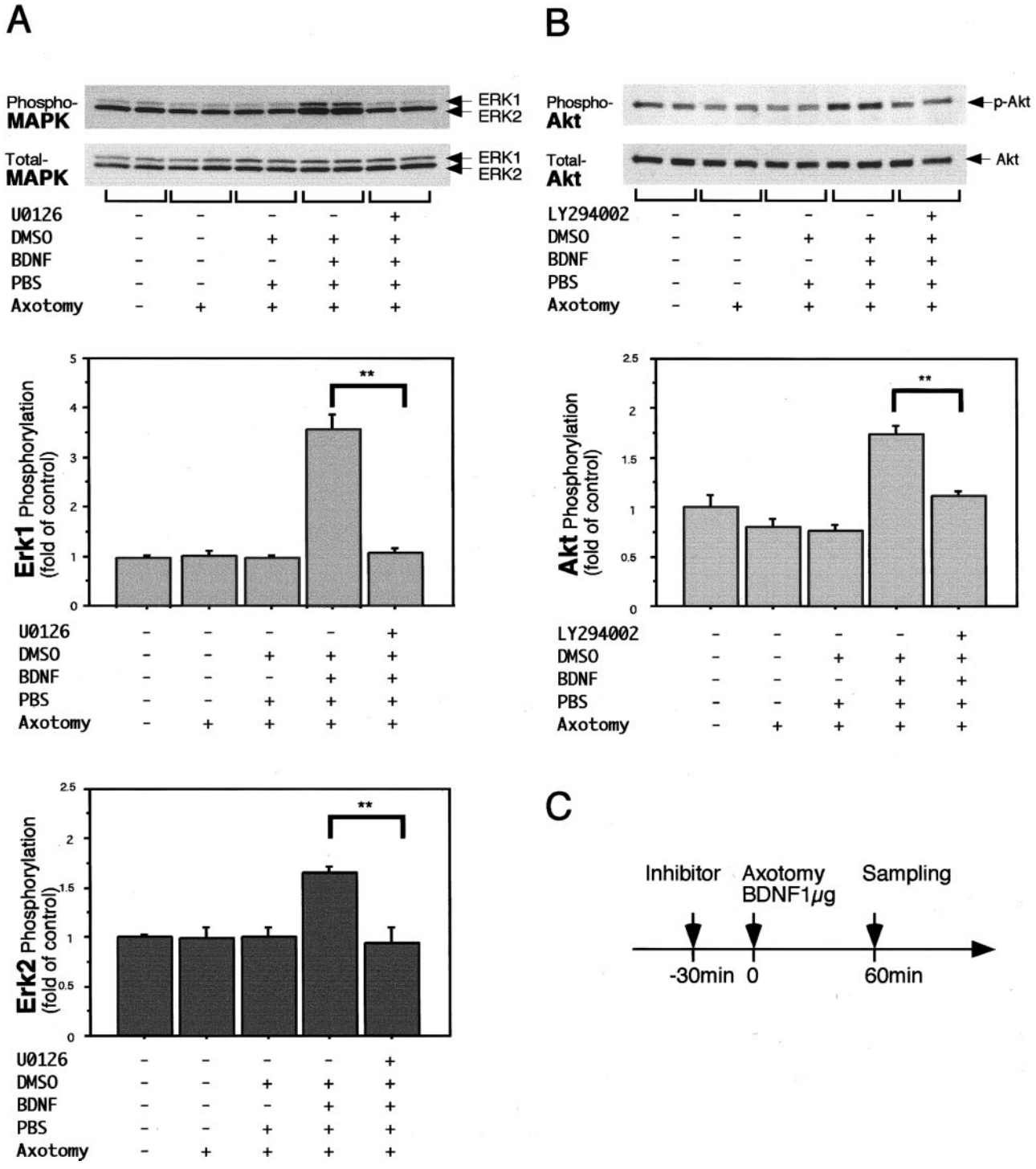

FIGURE 4. Inhibition of MAPK and Akt activity by each specific inhibitor. Assessments of the phosphorylation of MAPK and Akt by immunoblot are shown. A representative photograph of Western blot analysis ($n = 2$, *top panel*) and the optical densities of phosphorylated-MAPK (Erk1 or Erk2) (**A**) and Akt (**B**) by Western blot analysis are expressed as a ratio to those of nonphosphorylated MAPK and Akt ($n = 6$ or 4, *bottom panel*). Note the significant inhibitor-induced decrease in phosphorylated-MAPK and Akt on the axotomized retinal ganglion cells ($^{**}P < 0.01$, ANOVA followed by Scheffé post hoc test, each concentration dose, $n = 6$ or 4). (**C**) Experimental time course of drug and BDNF administration and sampling. Note that each specific inhibitor was injected intravitreously 30 minutes before administration of BDNF.

seemed necessary to treat the retina with signal-inhibiting drugs before the administration of BDNF.

In the developing RGCs, MAPK activation was found to be important not only for stimulating neuronal differentiation induced by neural cell adhesion molecule (NCAM), laminin (LN), and N-cadherin,[23,33] but also for neuronal growth and survival induced by various growth factors, including BDNF, ciliary neurotrophic factor (CNTF), and cAMP.[10,24,34] Activation of MAPK has also been observed in activated glia, mainly in the RMCs, after the systemic administration of α_2-adrenergic agonist,[35] light damage,[36] or intravitreous injection of neurotrophin[37] and axokine, an analogue of CNTF.[38] Thus, the activation of MAPK was consistently found in both RGCs and RMCs, after various developmental, neurodegenerative and/or neuro-

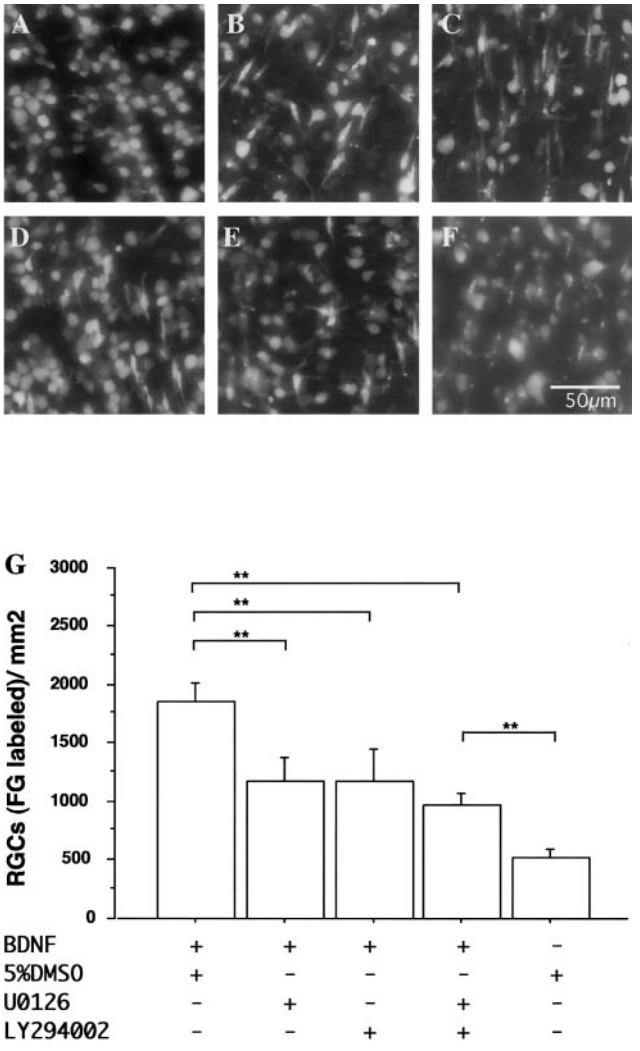

FIGURE 5. Representative photographs of fluorochrome-labeled flat-mounted retinas and the BDNF-induced activation of MAPK and Akt. Fluorochrome-labeled RGCs in corresponding regions of an unlesioned control retina (**A**), after axotomy at 10 days without (**B**) or with intravitreous administration of 5% DMSO-PBS (**C**), BDNF and 5% DMSO-PBS (**D**), 0.15 nmol per eye U0126 and BDNF (**E**), and 0.4 nmol per eye LY294002 and BDNF (**F**). (**G**) Quantitative analysis of rescued fluorochrome-labeled RGCs from the ON 10 days after axotomy is also shown. (**$P < 0.01$, ANOVA followed by the Scheffé post hoc test, each concentration dose $n = 7$ or 8.) Intravitreous coinjection of BDNF and MAPK and/or PI3K inhibitor decreased the BDNF-induced neuroprotective effects on the axotomized RGCs.

protective paradigms in the retina. Our result also indicated that the phosphorylated (activated) forms of MAPK were evident in RGCs and RMCs. In contrast to the MAPK route, PI3K generates various D3-phosphorylated phosphatidylinositides, which serve as second messengers leading to the activation of Akt. Active Akt has been shown to protect cells, in general, against apoptosis by phosphorylating Bad, which interferes with various steps of apoptosis including release of mitochondrial cytochrome-*c*,[39] suppression of caspase-9,[40] and activation of transcription factors including, FKHRL1, a member of the forkhead family,[41] and nuclear factor (NF)-κB, a transcription factor that promotes cell survival.[42] On the axotomized rat retina, BDNF[28] and IGF-1[27] have been shown to activate Akt and to suppress the cleavage and enzymatic activity of caspase-3, a neuronal cell death effector. In this examination, we demonstrated that BDNF-induced activation of Akt was evident in RGCs and amacrine cells in the GCL. In a general scheme, the caspase system could be a major route to cellular apoptosis; however, activation or inhibition of forkhead-type transcription factors such as FKHR, L1, and AFX, may also affect cell survival, because these factors are now known to be direct targets of Akt.[41,43]

Both MAPK and Akt pathways were activated on the axotomized and BDNF-stimulated RGCs, suggesting that TrkB of the RGCs may use the so-called Shc binding site to trigger the BDNF response in the activated RGCs, because the Shc binding site is the only site that may transmit signals to both the Ras-MAPK and PI3K-Akt routes.[12] Besides BDNF, many other growth factors, such as IGF-1,[27] glial cell–derived neurotrophic factor (GDNF),[44] platelet-derived growth factor (PDGF),[45] bFGF, and CNTF[36] can activate MAPK and/or PI3K pathways. Receptors for these growth factors are all tyrosine kinases, and all these receptor tyrosine kinases may transmit signals through Shc, a versatile phosphotyrosine adapter. However, accumulating evidence suggests that the Shc-related gene family containing three genes (Shc/ShcA, Sck/ShcB, N-Shc/ShcC)[13,14] and N-Shc/ShcC or Sck/ShcB may be functional in most neurons in the CNS.[13,14,46] We have also demonstrated in the retina that N-Shc/ShcC could be a potential phosphotyrosine adapter among the Shc family members for BDNF signaling and that the induction of N-Shc/ShcC phosphorylation by BDNF was transient and peaked at 1 hour after lesion.[16] Taken together, our data suggest that axotomized RGCs on BDNF stimulation activate N-Shc/ShcC, which leads to activation of both MAPK and Akt, leading ultimately to further downstream signaling for neuroprotection and/or neuroregeneration in the retina.

Acknowledgments

The authors thank Itsuko Nakano for excellent technical assistance, Takeshi Nakamura for comments on the manuscript; Yutaka Fukuda, Hajime Sawai, Toru Inoue (Graduate School of Medicine, Osaka University, Osaka, Japan) and Masami Watanabe (Institute for Developmental Research) for encouragement, support, and technical assistance with surgical procedures of optic nerve axotomy, retrograde labeling, and cell counting.

References

1. Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* 2000;14:2919–2937.
2. von Bartheld CS. Neurotrophins in the developing and regenerating visual system. *Histol Histopathol.* 1998;13:437–459.
3. Mansour-Robaey S, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. *Proc Natl Acad Sci USA.* 1994;91:1632–1636.
4. 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.
5. Watanabe M, Sawai H, Fukuda Y. Survival of axotomized retinal ganglion cells in adult mammals. *Clin Neurosci.* 1997;4:233–239.
6. Rocha M, Martins RA, Linden R. Activation of NMDA receptors protects against glutamate neurotoxicity in the retina: evidence for the involvement of neurotrophins. *Brain Res.* 1999;827:79–92.
7. Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci.* 1994;35:907–915.
8. LaVail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci.* 1998;39:592–602.
9. 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.

10. Shen S, Wiemelt AP, McMorris FA, Barres BA. Retinal ganglion cells lose trophic responsiveness after axotomy. *Neuron.* 1999;23:285–295.
11. Sommerfeld MT, Schweigreiter R, Barde YA, Hoppe E. Down-regulation of the neurotrophin receptor TrkB following ligand binding: evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. *J Biol Chem.* 2000;275:8982–8990.
12. Atwal JK, Massie B, Miller FD, Kaplan DR. The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron.* 2000;27:265–277.
13. Nakamura T, Sanokawa R, Sasaki Y, Ayusawa D, Oishi M, Mori N. N-Shc: a neural-specific adapter molecule that mediates signaling from neurotrophin/Trk to Ras/MAPK pathway. *Oncogene.* 1996;13:1111–1121.
14. Nakamura T, Muraoka S, Sanokawa R, Mori N. N-Shc and Sck, two neuronally expressed Shc adapter homologs: their differential regional expression in the brain and roles in neurotrophin and Src signaling. *J Biol Chem.* 1998;273:6960–6967.
15. Nakamura T, Komiya M, Gotoh N, Koizumi S, Shibuya M, Mori N. Discrimination between phosphotyrosine-mediated signaling properties of conventional and neuronal Shc adapter molecules. *Oncogene.* 2002;21:22–31.
16. Nakazawa T, Nakano I, Sato M, Nakamura T, Tamai M, Mori N. Comparative expression profiles of Trk receptors and Shc-related phosphotyrosine adapters during retinal development: potential roles of N-Shc/ShcC in BDNF signal transduction and modulation. *J Neurosci Res.* In press.
17. Dolcet X, Egea J, Soler RM, Martin-Zanca D, Comella JX. Activation of phosphatidylinositol 3-kinase, but not extracellular- regulated kinases, is necessary to mediate brain-derived neurotrophic factor-induced motoneuron survival. *J Neurochem.* 1999;73:521–531.
18. Encinas M, Iglesias M, Llecha N, Comella JX. Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *J Neurochem.* 1999;73:1409–1421.
19. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem.* 1999;274:22569–22580.
20. Qui MS, Green SH. PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. *Neuron.* 1992;9:705–717.
21. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science.* 1999;286:1358–1362.
22. Williams EJ, Doherty P. Evidence for and against a pivotal role of PI 3-kinase in a neuronal cell survival pathway. *Mol Cell Neurosci.* 2000;15:330–338.
23. Dimitropoulou A, Bixby JL. Regulation of retinal neurite growth by alterations in MAPK/ERK kinase (MEK) activity. *Brain Res.* 2000;858:205–214.
24. Meyer-Franke A, Wilkinson GA, Kruttgen A, et al. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron.* 1998;21:681–693.
25. Kermer P, Klöcker N, Labes M, Bähr M. Inhibition of CPP32-like proteases rescues axotomized retinal ganglion cells from secondary cell death in vivo. *J Neurosci.* 1998;18:4656–4662.
26. Kermer P, Klöcker N, Labes M, Thomsen S, Srinivasan A, Bähr M. Activation of caspase-3 in axotomized rat retinal ganglion cells in vivo. *FEBS Lett.* 1999;453:361–364.
27. Kermer P, Klöcker N, Labes M, Bähr M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 In vivo. *J Neurosci.* 2000;20:2–8.
28. Klöcker N, Kermer P, Weishaupt JH, Labes M, Ankerhold R, Bähr M. Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells In vivo does not exclusively depend on phosphatidyl-inositol-3′-kinase/protein kinase B signaling. *J Neurosci.* 2000;20:6962–6967.
29. Bähr M. Live or let die: retinal ganglion cell death and survival during development and in the lesioned adult CNS. *Trends Neurosci.* 2000;23:483–490.
30. Nakazawa T, Tomita H, Yamaguti K, et al. Neuroprotective effect of nipradilol on axotomized rat retinal ganglion cells. *Curr Eye Res.* in press.
31. Sawai H, Clarke DB, Kittlerova P, Bray GM, Aguayo AJ. Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from regenerating retinal ganglion cells. *J Neurosci.* 1996;16:3887–3894.
32. Schuettauf F, Naskar R, Vorwerk CK, Zurakowski D, Dreyer EB. Ganglion cell loss after optic nerve crush mediated through AMPA-kainate and NMDA receptors. *Invest Ophthalmol Vis Sci.* 2000;41:4313–4316.
33. Perron JC, Bixby JL. Distinct neurite outgrowth signaling pathways converge on ERK activation. *Mol Cell Neurosci.* 1999;13:362–378.
34. Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron.* 1995;15:805–819.
35. Peng M, Li Y, Luo Z, Liu C, Laties AM, Wen R. α_2-Adrenergic agonists selectively activate extracellular signal–regulated kinases in Muller cells in vivo. *Invest Ophthalmol Vis Sci.* 1998;39:1721–1726.
36. Liu C, Peng M, Laties AM, Wen R. Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J Neurosci.* 1998;18:1337–1344.
37. Wahlin KJ, Campochiaro PA, Zack DJ, Adler R. Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. *Invest Ophthalmol Vis Sci.* 2000;41:927–936.
38. Peterson WM, Wang Q, Tzekova R, Wiegand SJ. Ciliary neurotrophic factor and stress stimuli activate the Jak-STAT pathway in retinal neurons and glia. *J Neurosci.* 2000;20:4081–4090.
39. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 1997;91:231–241.
40. Cardone MH, Roy N, Stennicke HR, et al. Regulation of cell death protease caspase-9 by phosphorylation. *Science.* 1998;282:1318–1321.
41. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell.* 1999;96:857–868.
42. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signaling. *Nature.* 1999;401:86–90.
43. Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J.* 2000;349:629–634.
44. Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, Comella JX. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. *J Neurosci.* 1999;19:9160–9169.
45. Franke TF, Yang SI, Chan TO, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* 1995;81:727–736.
46. Kojima T, Yoshikawa Y, Takada S, et al. Genomic organization of the Shc-related phosphotyrosine adapters and characterization of the full-length Sck/ShcB: specific association of p68-Sck/ShcB with pp135. *Biochem Biophys Res Commun.* 2001;284:1039–1047.