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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

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The neurotrophin brain-derived neurotrophic factor (BDNF) serves as a survival, mitogenic, and differentiation factor in both the developing and adult CNS and PNS. In an attempt to identify the molecular mechanisms underlying BDNF neuroprotection, we studied activation of two potentially neuroprotective signal transduction pathways by BDNF in a CNS trauma model. Transection of the optic nerve (ON) in the adult rat induces secondary death of retinal ganglion cells (RGCs). Repeated intraocular injections of BDNF prevent the degeneration of RGCs 14 d after ON lesion most likely by inhibition of apoptosis. Here, we report that BDNF activates both protein kinase B (PKB) via a phosphatidyl-inositol-3′-kinase (PI-3-K)-dependent mechanism and the mitogen-activated protein kinases extracellular signal-regulated kinase 1 (ERK1) and ERK2. Furthermore, we provide evidence that BDNF suppresses cleavage and enzymatic activity of the neuronal cell death effector caspase-3. Distinct from our recent study in which inhibition of the PI-3-K/PKB pathway attenuated the survival-promoting action of insulin-like growth factor-I on axotomized RGCs (Kermer et al., 2000), it does not in the case of BDNF. Thus, we assume that BDNF does not depend on a single signal transduction pathway exerting its neuroprotective effects on lesioned CNS neurons.

Key words: retinal ganglion cell; axotomy; caspase-3; brain-derived neurotrophic factor (BDNF); neuroprotection; mitogen-activated protein kinase (MAPK); protein kinase B (PKB)

Fiber tract lesions in the adult mammalian CNS are known to result in secondary degeneration of the injured neurons by apoptosis (Garcia-Valenzuela et al., 1994). Besides their action as mitogenic and differentiation factors in the developing nervous system, members of the nerve growth factor (NGF) gene family, referred to as the neurotrophins, have been shown to prevent this neuronal degeneration (for review, see Snider and Johnson, 1989; Lewin and Barde, 1996), most likely by hindering the execution of the death program within these cells. However, up to date, the actual mechanisms of neurotrophin-mediated neuroprotection *in vivo* remain elusive.

The cellular effects of neurotrophins are transmitted by activation of the high-affinity Trk tyrosine kinase receptors TrkA, TrkB, and TrkC, and the low-affinity neurotrophin receptor p75 (Barbacid, 1995). On binding to these receptors, neurotrophins are known to activate various intracellular signal transduction pathways, including the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidyl-inositol-3′-kinase/protein kinase B (PI-3-K/PKB) pathway, which appear to interfere with cell survival and cell death mechanisms (Segal and Greenberg, 1996; Nunez and del Peso, 1998). The relevance of the Ras/MAPK pathway for cell survival remains ambiguous, because several studies have either failed to demonstrate any role of this pathway in cell survival or have even shown death-promoting effects (Gunn-Moore et al., 1997; Pritchard and McMahon, 1997; Alessandrini et al., 1999). In contrast, it is now well established that activation of the PI-3-K/PKB pathway plays a central role in growth factor-mediated protection against neuronal apoptosis *in vitro* (Yao and Cooper, 1995; Nonomura et al., 1996; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Kulik et al., 1997; Shimoke et al., 1997). PI-3-K generates various D3-phosphorylated phosphatidylinositides, which serve as second messengers eventually leading to activation of PKB (Franke et al., 1997; Shimoke et al., 1997; Vanhaesebroeck et al., 1997). Besides its multiple functions in cell metabolism, active PKB has been shown to protect against apoptosis (for review, see Coffer et al., 1998). Most recently, Cardone et al. (1998) demonstrated *in vitro* that PKB can phosphorylate and thereby inhibit human caspase-9, an initiator of the neuronal cell death effector caspase-3. By analogy, PKB has also been shown to phosphorylate and thereby inactivate the pro-apoptotic protein Bcl-2-associated death protein (Bad) (Datta et al., 1997; del Peso et al., 1997).

Here we characterized the neuroprotective signaling pathway of brain-derived neurotrophic factor (BDNF) in an established model of CNS injury, inducing secondary death of retinal ganglion cells (RGCs) by transection of the optic nerve (ON) in the adult rat (Villegas-Perez et al., 1988, 1993). Our *in vivo* data demonstrate that BDNF, one of the most effective survival factors in this model (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Klöcker et al., 1998), activates both the PI-3-K/PKB and Ras/MAPK pathways in the retina, eventually suppressing axotomy-induced caspase-3 activity.

MATERIALS AND METHODS

Animal surgery. Adult female Sprague Dawley rats (Charles River, Sulzfeld, Germany) were anesthetized by intraperitoneal injection of chloral hydrate (0.42 gm/kg bodyweight). The right ON was transected as described in detail previously (Klöcker et al., 1998). Briefly, the right orbita was opened saving the supraorbital vein, and the lacrimal gland was subtotally resected. By means of a small retractor, the superior extraocular muscles were spread, and the ON was exposed after longitudinal incision of the eye retractor muscle and the perineurium. The ON was transected ~2 mm from the posterior pole of the eye taking care not to damage the retinal blood supply. The latter was checked by fundoscopy after surgery.

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Retrograde labeling of RGCs. To determine RGC densities, cells were retrogradely labeled with the fluorescent tracers Fast Blue (FB) (Dr. Illing Chemie, Gross-Umstadt, Germany) and Fluorogold (FG) (Fluorochrome Inc., Englewood, CO). For FB and FG staining, a small piece of gel foam soaked in 2% aqueous FB or 5% aqueous FG was placed at the ocular stump of the ON after axotomy.

Drug administration. Recombinant human BDNF (Alomone Labs, Jerusalem, Israel) was dissolved in a 1% solution of bovine serum albumin in PBS at a concentration of 250 ng/μl. The irreversible inhibitor of caspase-3-like caspases, benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethylketone (z-DEVD-cmk) (Bachem, Heidelberg, Germany) was dissolved in 2% DMSO (Sigma, Deisenhofen, Germany) in PBS at a concentration of 2000 ng/μl. Stock solutions of the PI-3-K inhibitors wortmannin (WM) (Sigma) and LY294002 (Biomol Research Laboratories, Hamburg, Germany) were prepared in 100% DMSO at 1 and 100 mM, respectively. For lower concentrations, we used respective dilutions in PBS. A stock solution of the MAPK/ERK kinase-1 (MEK-1) inhibitor PD98059 (New England Biolabs GmbH, Schwalbach, Germany) was prepared in 100% DMSO at a concentration of 50 mM. For lower concentrations, we used respective dilutions in DMSO. For intraocular injection of the above drugs, animals were anesthetized by diethylether. By means of a glass microelectrode with a tip diameter of 30 μm, 2 μl of the respective solution was injected into the vitreous space puncturing the eye at the cornea–sclera junction.

We used two different intraocular treatment regimens: (1) for the neuroprotection studies, BDNF and additional drugs were injected on days 4, 7, and 10 after axotomy, except for z-DEVD-cmk, which was injected on days 0, 4, 7, and 10 after lesion; (2) for all Western blot studies and for the caspase-3 activity assays, agents were injected on days 0 and 4 after lesion. Protein lysates were prepared 6 hr after the last injection.

RGC densities. Fourteen days after ON transection, animals received an overdose of chloral hydrate, and the eyes were removed. The retinas were dissected, flat-mounted on glass slides, and fixed in 4% paraformaldehyde in PBS for 20 min. They were examined by fluorescence microscopy (Axiovert 35; Zeiss, Göttingen, Germany) using a UV filter (365/397 nm) for FB and FG fluorescence. RGC densities were determined by counting tracer-labeled RGCs in 12 distinct areas of 62,500 μm^2 each (three areas per retinal quadrant at three different eccentricities of one-sixth, one-half, and five-sixths of the retinal radius) (Klöcker et al., 1998). Cell counts were done in duplicate by two independent investigators.

Caspase activity assay. For the caspase-3 fluorogenic activity assay, animals received an overdose of chloral hydrate, and both eyes were removed on day 4 after lesion 6 hr after the last intraocular drug treatment. Untreated contralateral retinas served as controls. Retinas were homogenized, lysed (150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM EDTA, and 1% Triton, containing 0.1 mM PMSF, and 2 μg/ml pepstatin, leupeptin, and aprotinin) for 10 min at 37°C, and cell debris was pelleted at 14,000 × *g* for 15 min. The protein concentration of the supernatant was determined using the BCA reagent (Pierce, Rockford, IL). Fresh protein lysates (30 μl) were incubated with 100 μM DEVD-AMC (Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin), a fluorogenic substrate of caspase-3-like caspases. Caspase activity was determined measuring optical density (OD) units every 15 min for 2 hr using 360 nm excitation and 480 nm emission wavelength (CytoFlour 2350) and calculated as the increase in OD per microgram of protein over time (Kermer et al., 1999a).

Western blotting. Western blot experiments to determine the expression of caspase-3, PKB, MAPK, and their active forms were performed on day 4 after ON transection ~6 hr after the last intraocular drug treatment. Retinas were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1 mM PMSF, and 2 μg/ml pepstatin, leupeptin, and aprotinin, pH 8.3. The cell suspension was lysed on ice for 20 min, and cell debris was pelleted at 14,000 × *g* for 15 min. The protein concentration of the supernatant was determined using the BCA reagent (Pierce). After separation by reducing SDS-PAGE (Ausubel et al., 1987) of the lysates, proteins were transferred to a polyvinylidene difluoride membrane and blocked with 5% skim milk in 0.1% Tween 20/PBS (PBS-T). The membranes were incubated with the respective primary antibodies (in 1% skim milk in PBS-T) for 1 hr at room temperature (RT). After washing in PBS-T, the membranes were incubated with HRP-conjugated secondary antibodies against rabbit IgG or against goat IgG for 1 hr at RT (1:2000 in PBS-T; Dianova, Hamburg, Germany). Labeled proteins were detected using the ECL-plus reagent (Amersham, Arlington Heights, IL) following the instructions of the supplier. Densitometric analysis of protein bands was performed using TINA 2.0 software; density values in the various experimental groups were calculated as percentage of the contralateral control groups. The following primary antibodies were used: (1) anti-PKB (1:1000; New England Biolabs GmbH); (2) anti-phospho-PKB (1:1000; New England Biolabs GmbH); (3) anti-MAPK (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA); (4) anti-phospho-MAPK (1:5000; Promega, Madison, WI); and (5) anti-caspase-3 (CM1; 1:1000; IDUN Pharmaceuticals, La Jolla, CA).

Statistics. Data are given as mean ± SEM unless stated otherwise. Statistical significance was assessed using one-way ANOVA followed by Duncan's *post hoc* test.

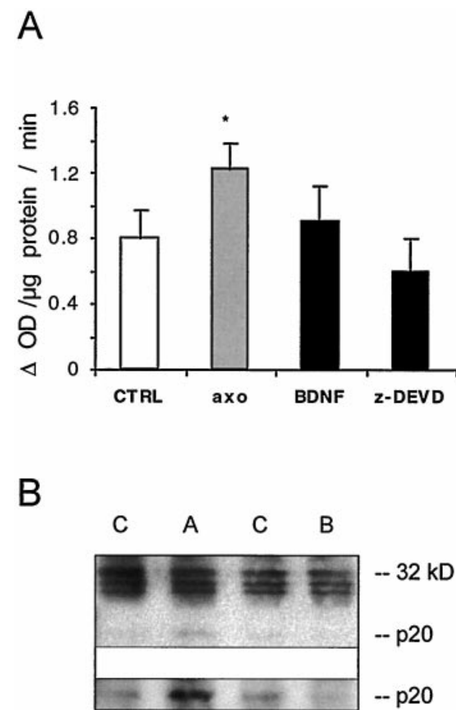

Figure 1. BDNF inhibits caspase-3 activity. *A*, Caspase-3 activity in total retinal tissue as determined by DEVD-AMC cleavage 4 d after ON transection. Data in the experimental groups are given as mean ± SD percentage of caspase-3 activity in contralateral control retinas. **p* < 0.05, statistically significant from control. *CTRL*, Unlesioned contralateral control; *axo*, axotomy without treatment; *BDNF*, axotomy with additional BDNF treatment on days 0 and 4 after lesion; *z-DEVD*, axotomy with additional z-DEVD-cmk treatment on days 0 and 4 after lesion. *B*, Western blot for caspase-3 applying the CM-1 antibody. To better visualize the active p20 fragment, a longer exposure is shown in the *bottom panel*. *C*, Control; *A*, axotomy without treatment; *B*, axotomy with additional BDNF treatment on days 0 and 4 after lesion.

RESULTS

BDNF suppresses caspase-3 activity

Based on the hypothesis that BDNF exerts its neuroprotective effects via inhibition of apoptosis, we first investigated whether BDNF affects activation of the cell death effector caspase-3 in axotomized RGCs (Kermer et al., 1998, 1999a). To this end, we measured caspase activity levels by cleavage of the fluorogenic substrate Ac-DEVD-AMC known to be processed by caspase-3-like proteases. Activities in total retinal tissue 4 d after axotomy with and without BDNF treatment were compared. This time point was chosen because we had shown previously that a significant increase in caspase-3 activity after ON transection first occurs on day 4 (Kermer et al., 1999a, 2000). As shown in Figure 1, BDNF prevented the induction of caspase-3 activity after ON transection. The same effect could be achieved by intraocular treatment with the rather selective and irreversible inhibitor of caspase-3-like caspases z-DEVD-cmk. In line with these results, we found that BDNF and caspase-3 inhibition are not additive in promoting the survival of axotomized RGCs (Table 1). ON transection leads to retrograde death of ~85% of adult rat RGCs within 14 d after lesion. Three intraocular injections of 500 ng of BDNF on days 4, 7, and 10 after axotomy significantly enhanced RGC survival. Thirty-two percent of RGCs that would otherwise have died 14 d after lesion were rescued by treatment with BDNF alone (889 ± 47 cells/mm^2; $n = 4$). Additional intraocular administration of z-DEVD-cmk on days 0, 4, 7, and 10 after lesion, although being highly neuroprotective when applied alone (Kermer et al., 1998), did not significantly add to BDNF-mediated neuroprotection (905 ± 153 RGCs/mm^2; $n = 4$). To further strengthen the hypothesis that BDNF inactivates caspase-3, we performed Western blot

Table 1. Effects of single and combined intraocular treatment with BDNF, z-DEVD-cmk, wortmannin, LY294002, and PD98059 on RGC survival 14 d after optic nerve transection in the adult rat

	⅙ Retinal radius	3/6 Retinal radius	5/6 Retinal radius	Average
Normal control	2647 ± 95	2289 ± 53	1180 ± 46	2039 ± 56 (n = 11)
Axotomy w/o th.	409 ± 50	354 ± 56	254 ± 49	339 ± 43 (n = 6)
Vehicle	587 ± 75	394 ± 90	255 ± 77	412 ± 77 (n = 4)
BDNF 500	1253 ± 138	777 ± 80	382 ± 61	889 ± 47* (n = 4)
z-DEVD-cmk 4000	1278 ± 137	988 ± 107	558 ± 57	942 ± 99* (n = 4)
BDNF + z-DEVD-cmk 4000	1227 ± 228	1038 ± 182	451 ± 56	905 ± 153* (n = 4)
BDNF + wortmannin	1116 ± 64	971 ± 85	599 ± 42	895 ± 61* (n = 4)
BDNF + LY294002	1259 ± 42	898 ± 63	657 ± 99	938 ± 72* (n = 3)
BDNF + PD98059	999 ± 116	933 ± 82	598 ± 15	843 ± 68* (n = 4)

Data are given as mean ± SEM RGC densities (cells per square millimeter) at one-sixth, one-half, and five-sixths of the retinal radius and averaged over the radius. Axo w/o th., Axotomy without treatment; BDNF 500, intraocular injections of 500 ng of BDNF on days 4, 7, and 10 after lesion; z-DEVD-cmk 4000, intraocular injections of z-DEVD-cmk of 4000 ng on days 0, 4, 7, and 10 after lesion; BDNF + wortmannin, intraocular injections of 0.1 mM wortmannin; BDNF + LY294002, intraocular injections of 0.1 mM LY294002; BDNF + PD98059, intraocular injections of 5 mM PD98059. Intraocular injections of wortmannin, LY294002, and PD98059 were combined with intraocular injections of BDNF on days 4, 7, and 10 after lesion. Statistical significance was assessed using one-way ANOVA, followed by Duncan's *post hoc* test (* $p < 0.05$).

analysis using an antibody that recognizes both inactive procaspase-3 and the cleaved p20 fragment resulting from caspase-3 activation. These experiments revealed that the increase in p20 expression observed in axotomized but otherwise untreated retinas was suppressed by BDNF. No significant differences in total retinal caspase-3 protein expression could be detected in any experimental retina compared with its respective unlesioned contralateral control retina (Fig. 1). This is in good agreement with previous reverse transcription-PCR results showing no changes in caspase-3 mRNA expression up to 6 d after axotomy (Kermer et al., 1999a).

BDNF activates both the PI-3-K and MAPK signaling pathways

In an attempt to identify the neuroprotective signal transduction pathways activated by BDNF, we studied retinal expression of PKB and ERK1/2 (p42/p44 MAPK) and their degree of phosphorylation, known to be crucial for their activation.

Whereas ON transection alone led to a decrease in phospho-PKB expression (49 ± 9%; $n = 3$), additional BDNF treatment induced phosphorylation of PKB above control levels (229 ± 38%; $n = 4$) (Fig. 2). BDNF-mediated activation of PKB depended on active PI-3-K, because the PI-3-K inhibitors wortmannin and LY294002 abolished it (Fig. 2).

The same results were obtained for retinal expression of phosphorylated p44 and p42 MAPK. Although we found decreased levels 4 d after ON transection without treatment (71 ± 19 and 50 ± 16% for p44 and p42, respectively; $n = 3$), there was a strong increase in phosphorylation of p44 and p42 MAPK after BDNF treatment (310 ± 38 and 249 ± 45%, respectively; $n = 4$) (Fig. 3).

Vehicle treatment did not mimic these effects, on neither PKB nor ERK1/2 phosphorylation. Expression of the unphosphorylated PKB and MAPK proteins remained constant in the various experimental groups.

Effects of PI-3-K and MEK inhibitors on BDNF neuroprotection

In a previous study, we could demonstrate that insulin-like growth factor I (IGF-I) protects axotomized RGCs by a mechanism involving PI-3-K-dependent activation of PKB (Kermer et al., 2000). Given the present data, which are strongly suggestive of the idea that BDNF uses the same signaling pathway for its neuroprotective effects, we addressed the question as to whether selective inhibition of PI-3-K would reduce BDNF neuroprotection in our trauma model. Intriguingly, despite reducing BDNF-mediated activation of PKB (Fig. 2), intraocular administration of neither wortmannin nor LY294002 attenuated the survival-promoting effects of BDNF (Table 1). We then tried to block BDNF-induced ERK1/2 phosphorylation by inhibiting upstream kinases. To this end, we first injected the MEK-1 inhibitor PD98059 at two different concentrations of 5 and 50 mM. For an estimated vitreous chamber volume of the adult rat of 60 μl, these injections resulted in a final concentration of ~85 and 850 μM, respectively. This is up to two orders of magnitude higher than the IC_{50} of PD98059 *in vitro* (5–10 μM). However, we could not observe a significant reduction in the basal expression of phospho-ERK1/2 in control retinas or in the BDNF-induced upregulation of phospho-ERK1/2. In line with that, BDNF neuroprotection was not attenuated by additional intraocular injections of PD98059 (Fig. 3, Table 1).

Similarly, intraocular injection of the MEK inhibitor U0126 (Calbiochem GmbH, Bad Soden, Germany) at concentrations 100 times greater than the IC_{50} *in vitro* (50–60 nM) did not lower the level of phosphorylated ERK1/2 expression induced by BDNF (data not shown).

DISCUSSION

Our *in vivo* study was designed to further characterize the neuroprotective signaling pathways of the neurotrophin BDNF in CNS trauma. The data demonstrate for the first time that BDNF activates PKB via a PI-3-K-dependent mechanism, as well as the MAP kinases ERK1 and ERK2 *in vivo*, eventually leading to inhibition of caspase-3. Despite the fact that activation of PKB has proven neuroprotective in the same trauma model (Kermer et al., 2000), it is not required however for the survival-promoting effects observed with BDNF.

BDNF has repeatedly been shown to promote survival of rat

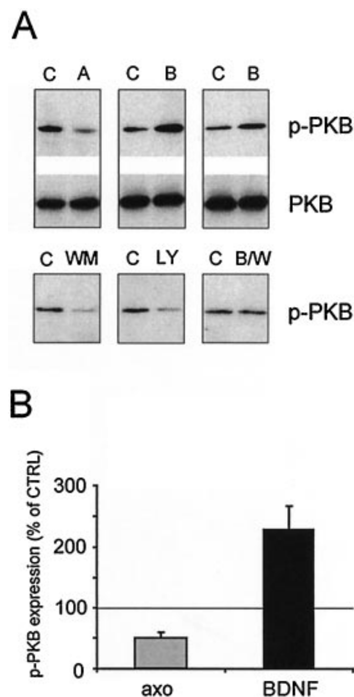

Figure 2. BDNF upregulates the expression of phosphorylated PKB via a PI-3-K-dependent mechanism. *A*, Western blot experiments applying anti-PKB and anti-phospho-PKB antibodies were performed using total retinal tissue 4 d after lesion in the following experimental groups: *A*, axotomy without treatment; *B*, axotomy with additional BDNF treatment on days 0 and 4 after lesion; *WM*, two intraocular injections of 0.1 mM wortmannin (2 μl) on days 0 and 4 in normal control retinas; *LY*, two intraocular injections of 1 mM LY294002 (2 μl) on days 0 and 4 in normal control retinas; *B/W*, combined intraocular injections of BDNF and wortmannin on days 0 and 4 after lesion; *C*, control. In each case, protein expression in the experimental groups was compared with that in the contralateral control retina. Representative blots from several experiments are shown. *B*, Relative expression levels of phosphorylated PKB in retinas 4 d after lesion without further treatment (*axo*; $n = 3$) and in retinas 4 d after ON transection and additional BDNF treatment (*BDNF*; $n = 4$) compared with the contralateral control retinas (normalized to 100%). Data are given as mean ± SEM.

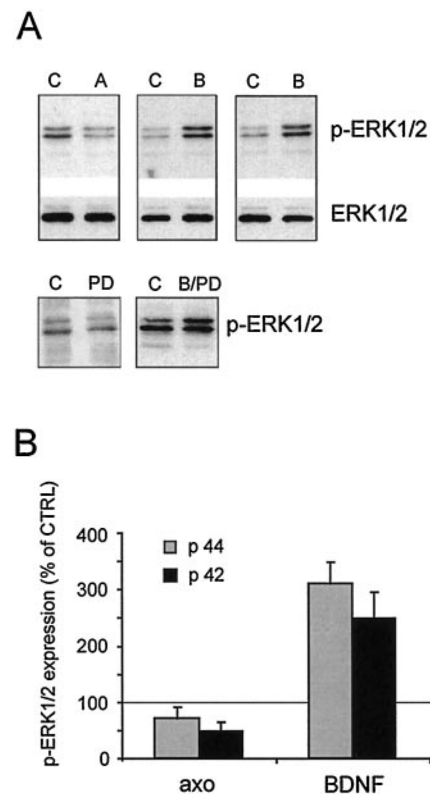

Figure 3. BDNF upregulates the expression of phosphorylated ERK1 and ERK2. *A*, Western blot experiments applying anti-ERK1/2 and anti-phospho-ERK1/2 were performed using total retinal tissue 4 d after lesion in the following experimental groups: *A*, axotomy without treatment; *B*, axotomy with additional BDNF treatment on days 0 and 4 after lesion; *PD*, two intraocular injections of 50 mM PD98059 (2 μl) on days 0 and 4 in normal control retinas; *B/PD*, combined intraocular injections of BDNF and PD98059 on days 0 and 4 after lesion; *C*, control. In each case, protein expression in the experimental groups was compared with that in the contralateral control retina. Representative blots from several experiments are shown. *B*, Relative expression levels of phosphorylated ERK1 and ERK2 in retinas 4 d after lesion without further treatment (*axo*; $n = 3$) and in retinas 4 d after ON transection and additional BDNF treatment (*BDNF*; $n = 4$) compared with the contralateral control retinas (normalized to 100%). Data are given as mean ± SEM.

RGCs both *in vitro* and *in vivo* (Johnson et al., 1986; Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Meyer-Franke et al., 1995; Peinado-Ramon et al., 1996; Klöcker et al., 1998). Experimental evidence suggests that BDNF hinders the induction of apoptosis (Cui and Harvey, 1995), but the actual mechanism of action has not yet been completely resolved. Our recent work ascribes caspase-3 a central role in the mediation of secondary RGC death after axonal lesion. Thus, we found increased cleavage of caspase-3 in retinas after ON transection in Western blot experiments, which localized immunohistochemically to RGCs (Kermer et al., 1998, 1999a). Inhibition of caspase-3 activity significantly promoted survival of axotomized RGCs (Kermer et al., 1998) approximately to the same extent as BDNF did in the present study. Intriguingly, in the long-term, both caspase-3 inhibition and BDNF treatment could not prevent but only delay the process of secondary degeneration of axotomized RGCs, independent of whether the treatment consisted of repeated intraocular injections or continuous supply with the neuroprotective agent via adenoviral gene transfer (Mansour-Robaey et al., 1994; Di Polo et al., 1998; Kermer et al., 1999b). These striking similarities in their effects on RGCs led us to investigate whether BDNF and caspase-3 inhibitors possibly use a common mechanism in their neuroprotective action. Indeed, intraocular injection of BDNF suppressed the increased expression of active caspase-3 as shown in Western blot experiments and reduced caspase-3 activity levels to control levels determined in a fluorogenic activity assay. These data are supported by our finding that simultaneous BDNF treatment and caspase-3 inhibition are not additive in promoting survival of axotomized RGCs *in vivo*.

A potential link between activation of receptor tyrosine kinases, including the BDNF high-affinity receptor TrkB, and inhibition of caspase-3 can be derived from *in vitro* studies, showing that growth factor-mediated neuroprotection on cerebellar granule cells depends on PI-3-K and subsequent activation of PKB (Nonomura et al., 1996; Shimoke et al., 1997; Skaper et al., 1998). So far, identified downstream targets of PKB are the initiator caspase-9, known to activate caspase-3, and the pro-apoptotic protein Bad. Both are inhibited upon phosphorylation by PKB *in vitro* (Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998). We therefore investigated whether ON lesion or BDNF treatment affect retinal expression of activated PKB. Four days after ON transection, a time point when most of the axotomized RGCs are still alive without further treatment (Peinado-Ramon. et al., 1996), we found a decrease in retinal expression of phosphorylated PKB. Additional BDNF treatment, however, strongly induced activation of PKB above control levels. Because the retinal expression of unphosphorylated PKB protein remained unchanged in response to either lesion or BDNF, the observed changes in expression of the phosphorylated protein must correspond to changes in PKB activity.

Rat RGCs receive trophic support from their main target of innervation, the superior colliculus. Thus, interruption of the retrograde axonal transport could account for the decreased level of PKB activity after ON transection. Such a hypothesis is on the other hand cut back by findings that the dependence of RGCs on retrograde trophic support diminishes with postnatal development

(Carpenter et al., 1986) and that the expression of trophic factors is upregulated within the retina in response to ON lesion (Gao et al., 1997) (Hirsch et al., 2000). Moreover, one should keep in mind that PKB activity will not only be regulated by trophic factors, because PKB is involved in a number of physiological processes, including intermediary metabolism and protein synthesis (Coffer et al., 1998). Nonetheless, the BDNF-induced increase in PKB activity, which depends on active PI-3-K, is in good agreement with our data on IGF-I neuroprotection in the same model system (Kermer et al., 2000). Unexpectedly and in contrast to IGF-I, however, BDNF neuroprotection was not affected when activation of PKB was suppressed by PI-3-K inhibitors. We conclude that BDNF, although activating the PI-3-K/PKB pathway, which proved to be neuroprotective in our model (Kermer et al., 2000), does not entirely depend on it to exert its RGC survival-promoting action. Therefore, we investigated an alternative signal transduction pathway known to be activated by TrkB, the Ras/MAPK pathway. As observed for PKB, ON transection decreased levels of phosphorylated ERK1 and ERK2 compared with the expression of the unphosphorylated proteins. Additional BDNF treatment induced activation of ERK1 and ERK2 above control levels. ERK1 and ERK2, prototypic members of the MAPK family, can be activated by various growth factors, including neurotrophins, through a pathway involving the respective tyrosine kinase receptors, the GTP-binding protein Ras, the kinase Raf-1, and MEKs (Blenis, 1993; Crews and Erikson, 1993; Davis, 1993). MAPKs have been ascribed mostly important roles in the regulation of cell growth and morphological differentiation (for review, see Fukunaga and Miyamoto, 1998). Studies on the role of MAPK for neuronal survival have been controversial (Gunn-Moore et al., 1997; Pritchard and McMahon, 1997; Skaper et al., 1998; Alessandrini et al., 1999; Bonni et al., 1999). Very recently, activated MAPK has been found to promote survival of postnatal rat RGCs both *in vitro* and *in vivo* (Meyer-Franke et al., 1998; Shen et al., 1999). These authors further reported that the MEK inhibitor PD98059 significantly attenuated survival of postnatal RGCs. In contrast, we found PD98059 at a concentration up to two orders of magnitude greater than the published IC_{50} *in vitro* neither effective in preventing BDNF-induced MAPK phosphorylation nor in attenuating the survival-promoting action of BDNF on lesioned RGCs in the adult animal. PD98059 is a strong inhibitor of MEK1 and a weaker one of MEK2, both known to be upstream activators of ERK1 and ERK2. Given that PD98059 shows similar diffusion properties in both postnatal and adult eyes, one could speculate that there exist other MEKs that phosphorylate ERK1/2. Their expression could be developmentally regulated, accounting for the different effects of PD98059 on the survival of postnatal and adult RGCs (Brott et al., 1993; Shen et al., 1999). Besides that, one has to consider that both MEK inhibitors we used in our experiments are highly sensitive to light (information of the suppliers). In contrast to intraocular injection in postnatal rats before eye-opening, injection of them into the vitreous of adult rat inevitably results in light exposure, which might affect their efficacy to block MEK activity. Moreover, it should be noted that, in response to strong MEK activators such as NGF in PC12 cells, PD98059 has been reported to leave some residual MEK activity, which could still be sufficient to activate the MAPK cascade (Alessi et al., 1995). It is very likely that BDNF represents a strong MEK activator in the retina. Free radical species generated by nitric oxide (NO) donors can trigger the activation of both the PI-3-K/PKB and Ras/MAPK pathways (Deora et al., 1998). In light of our studies demonstrating that BDNF upregulates retinal NO synthase activity in the adult rat (Klöcker et al., 1998, 1999), one could speculate that BDNF disposes of at least two, possibly even amplifying ways to induce its signaling events, which is the activation of the discussed pathways (1) via activation of TrkB and (2) via direct redox activation of Ras by increased NO synthesis.

In summary, our data reveal that BDNF activates at least two different signal transduction pathways in the adult rat retina, the PI-3-K/PKB and the Ras/MAPK pathways, which might directly or indirectly inhibit the activation of cell death mechanisms. There seems to be some redundancy in the contribution of these pathways to the survival-promoting action of BDNF on axotomized RGCs, because inhibition of PI-3-K did not attenuate the BDNF effects on RGC survival. Our study provides further insight into the mechanisms of BDNF neuroprotection, one of which is the suppression of caspase-3 activity. Because caspase-3 turns out to be the crucial executioner of apoptotic death in neurons (Nicholson and Thornberry, 1997), regulation of its activity promises to be a powerful tool in controlling neuronal cell survival. Whether retinal caspase-3 activity, however, is under direct regulatory control of BDNF signal transduction or whether BDNF-mediated inactivation of certain initiator caspases, i.e., caspase-8 or caspase-9, prevents caspase-3 activation is subject to current investigation.

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