

Brimonidine promotes axon growth after optic nerve injury through Erk phosphorylation

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It is well known that axons of the adult mammalian central nervous system have a very limited ability to regenerate after injury. Therefore, the neurodegenerative process of glaucoma results in irreversible functional deficits, such as blindness. Brimonidine (BMD) is an alpha2-adrenergic receptor agonist that is used commonly to lower intraocular pressure in glaucoma. Although it has been suggested that BMD has neuroprotective effects, the underlying mechanism remains unknown. In this study, we explored the molecular mechanism underlying the neuroprotective effect of BMD in an optic nerve injury (ONI) model. BMD treatment promoted optic nerve regeneration by inducing Erk1/2 phosphorylation after ONI. In addition, an Erk1/2 antagonist suppressed BMD-mediated axonal regeneration. A gene expression analysis revealed that the expression of the neurotrophin receptor gene *p75* was increased and that the expression of the tropomyosin receptor kinase B (*TrkB*) gene was decreased after ONI. BMD treatment abrogated the changes in the expression of these genes. These results indicate that BMD promotes optic nerve regeneration via the activation of Erk1/2.

Cell Death and Disease (2013) 4, e763; doi:10.1038/cddis.2013.298; published online 8 August 2013

Subject Category: Neuroscience

Spontaneous regeneration of lesioned axons is very limited in the adult mammalian central nervous system (CNS). The regeneration failure in the adult CNS might be attributed to the gradual decline in the intrinsic growth ability of neurons and the unfavorable extrinsic environment, which includes myelin-derived inhibitory molecules. Because of this lack of appropriate axonal regeneration, damage to the optic nerve causes permanent neuronal deficits, which are associated with the loss of vision.

Brimonidine (BMD) is a selective alpha2-adrenergic receptor agonist that is known for its role in reducing intraocular pressure (IOP) in the treatment of ocular hypertension and glaucoma.¹ Several clinical studies have reported its safety in lowering IOP;^{2–4} moreover, recent experiments suggest that it has a neuroprotective effect after various types of injury in animal models, such as optic neuropathy including optic nerve injury (ONI),^{5,6} ischemia,^{7,8} and experimental glaucoma.^{9–11} The neuroprotective effect of BMD appears to be independent of its IOP-lowering properties. BMD reduces the loss of retinal ganglion cells (RGCs) in a rat glaucoma model by modulating NMDA receptor function.¹² However, the mechanism underlying the axonal-regrowth-promoting effect of BMD remains a significant question.

It has been suggested that BMD exerts its neuroprotective effects on RGCs via the upregulation of neurotrophic growth factors, such as FGF2^{13,14} and BDNF.¹⁵ We reported previously that the activation of the tropomyosin receptor kinase B (TrkB) receptor—a BDNF receptor—is required for

the promotion of optic nerve regeneration, as well as the inhibition of the myelin signaling pathway.¹⁶ These findings suggest that neurotrophin receptors have roles in axon regeneration after optic nerve crush injury. Thus, it would be of interest to determine whether BMD promotes optic nerve regeneration by regulating the expression of neurotrophin receptors.

The Trk and the p75 neurotrophin receptors are often expressed in the same cell. These receptors work in a coordinated manner to modulate the responses of neurons to neurotrophins. Trk receptors mediate positive signals, such as the promotion of neuronal survival and axonal growth, whereas p75 mediates both positive and negative signals.^{17,18} In some cases, p75 opposes the function of Trk receptors. We reported previously that the inhibition of the p75 neurotrophin receptor causes the activation of TrkB, leading to optic nerve regeneration.¹⁹ In this study, we assessed the manner via which BMD promotes optic nerve regeneration via neurotrophin signaling.

Results

BMD promotes optic nerve regeneration. We first examined the effect of BMD on RGCs in an optic nerve crush injury mouse model. The regenerating fibers of RGCs were traced by injecting cholera toxin subunit B (CTB) conjugated to Alexa Fluor 555 into the vitreous of the retina 12 days after the crush injury (Figure 1a). A particularly large number of

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Keywords: Brimonidine; axon regeneration; Erk

Abbreviations: IOP, intraocular pressure; TrkB, tropomyosin receptor kinase B; RGC, retinal ganglion cells; CTB, cholera toxin beta subunit; BMD, brimonidine; ONI, optic nerve injury

Received 10.4.13; revised 27.6.13; accepted 15.7.13; Edited by A Verkhratsky

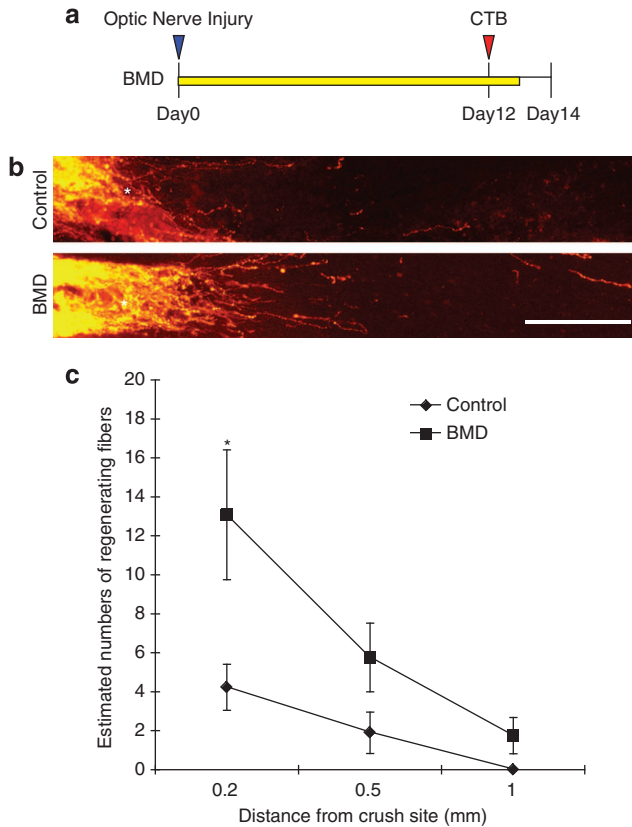


Figure 1 Brimonidine (BMD) promotes the regrowth of RGC axons after optic nerve injury. (a) Schematic representation of the experiment protocol used for optic nerve injury. (b) Longitudinal sections through the optic nerve showing CTB-labeled axons distal to the injury site in PBS-treated control and BMD-treated mice. *Injury site. Scale bar, 200 μm . (c) Quantitative analysis of regenerating axons extending 0.2, 0.5, and 1.0 mm from the end of the crush site at 14 days after injury. At least five different sections from each animal were quantified. Significant differences were noted between the PBS- and BMD-treated groups. $N = 7$ for each group. * $P < 0.05$; Student's *t*-test

CTB-labeled axons were observed in the optic nerve distal to the crush site in eyes that received BMD treatment (Figure 1b). A quantitative analysis showed that the mean estimated number of regenerating fibers was significantly increased at the 200 μm measurement point in mice treated with BMD compared with phosphate-buffered saline (PBS)-treated control mice (Figure 1c). However, at the 500 and 1000 μm measurement points, the mean estimated number of regenerating fibers was not significantly different between the two groups. These results indicate that BMD promotes the regeneration of RGC axons within a distance of 200 μm from the crush site.

Upregulation of TrkB and downregulation of p75 by BMD. A previous study revealed that BMD affected the expression of growth factors, neurotrophins, and their receptors, which are known to be involved in the regulation of neurite extension.^{20,21} Therefore, we examined whether the expression levels of these genes were changed by BMD treatment after optic nerve crush injury. In particular, we focused on the TrkB and p75 neurotrophin receptors.

We reported previously that activation of TrkB and loss of p75 promote optic nerve regeneration.^{16,19} Here, the relative mRNA levels of TrkB tended to decrease after ONI (Figure 2a). BMD treatment increased retinal TrkB expression 14 days after injury, whereas the expression levels of BDNF, the ligand for TrkB, were not increased (Figure 2b). p75 mRNA levels increased by fivefold 2 days after ONI in mice treated with PBS, and returned to normal levels by day 7 (Figure 2c). However, its levels were unaltered in mice treated with BMD. We also examined the expression level of TrkB and p75 proteins by western blotting. The results demonstrated that the expression profiles of these proteins are consistent with those of mRNA (Figure 2d and e). Further, the phosphorylation level of TrkB was also upregulated when TrkB protein expression was increased (Figure 2f). Thus, BMD treatment upregulated retinal TrkB at day 14 and suppressed the induction of p75 after ONI.

Further, we investigated whether the upregulation of TrkB by BMD was involved in optic nerve regeneration. After optic nerve crush injury, mice were administered K252a, a pan Trk inhibitor, intravitreally and were treated daily with BMD. A second injection of K252a was given 7 days after ONI (Figure 3a). BMD induced the phosphorylation of TrkB, whereas this was reduced after the administration of K252a (Figure 3b). This result demonstrated that intravitreal administration of K252a inhibited the activation of TrkB induced by BMD. However, a quantitative analysis revealed that the K252a treatment did not reduce the regrowth-promoting effect of BMD (Figures 1c, 3c, and 3d).

BMD stimulates retinal Erk1/2 activation after ONI.

TrkB-mediated neuroprotection involves several signaling pathways. PI3K/Akt and MEK/MAPK are the major intracellular signals of this effect.¹⁸ To determine whether these downstream molecules are involved in the BMD-induced TrkB-mediated growth of transected RGC axons, we examined the levels of active Erk 1/2 and Akt after optic nerve lesion. For this purpose, we performed western blot analyses of whole retinal homogenates collected 14 days after ONI, with or without BMD treatment. The results demonstrated that axotomized retinas treated with BMD exhibited a statistically significant increase in phospho-Erk1/2 compared with intact (N: no injury) or axotomized (I: ONI) retinas (Figures 4a and b). In contrast, the levels of phospho-Akt remained unchanged after ONI, in the presence or absence of BMD (Figures 4c and d).

Inhibition of BMD-induced optic nerve regeneration by an Erk antagonist.

To investigate whether Erk1/2 activation is required for RGC axonal regrowth after injury, we used the MEK inhibitor U0126. After optic nerve crush injury, mice were injected intravitreally with U0126 and were treated daily with BMD. A second injection of U0126 was given 7 days after ONI (Figure 5a). The administration of U0126 attenuated the regenerative effect mediated by BMD treatment (Figure 5b). A quantitative analysis revealed that the injection of U0126 reduced the regrowth-promoting effect of BMD significantly (Figures 1c and 5c). Taken together, these results indicate that the inhibition of Erk1/2 suppresses BMD-induced optic nerve regeneration, suggesting that Erk1/2 are key signaling molecules that regulate BMD-mediated RGC axonal growth.

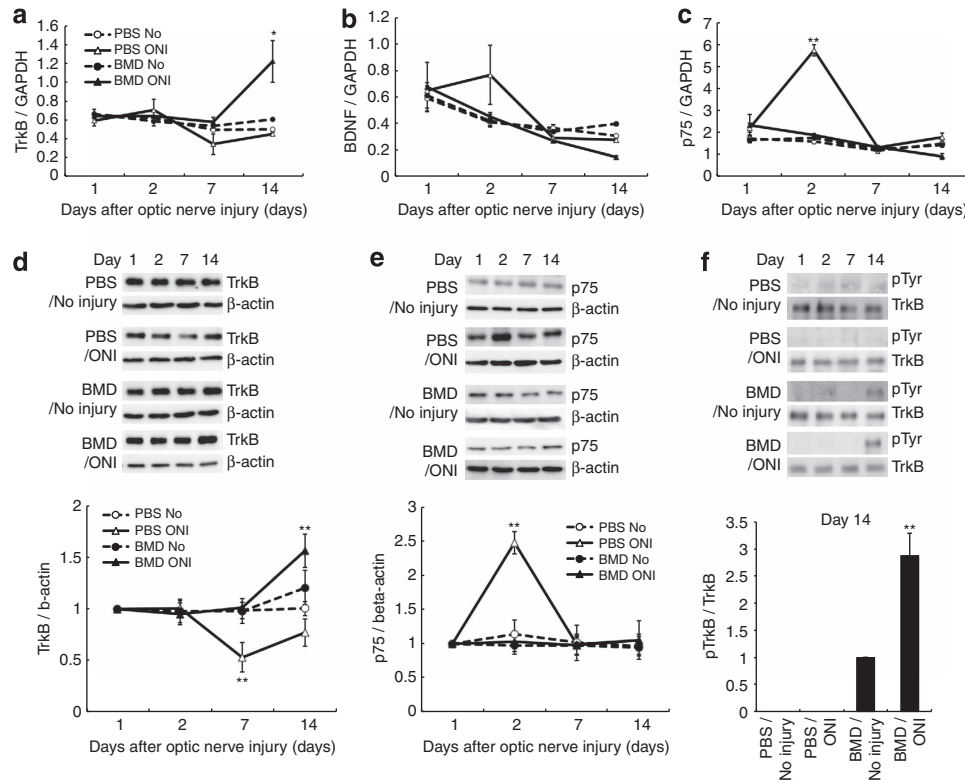


Figure 2 Quantification of RNA levels in PBS- or brimonidine-treated retinas after optic nerve injury. (a–c) Relative mRNA levels were measured using real-time PCR. Total RNA from PBS- or brimonidine-treated retinas was analyzed 1, 2, 7, and 14 days after optic nerve injury (ONI). (a) TrkB, (b) BDNF, and (c) p75 RNA levels in PBS- and brimonidine-treated retinas. The relative expression levels were determined by normalization to the level in the intact retina. $N = 3–4$. (d and e) Western blotting showing the temporal expression profiles of TrkB (d) and p75 (e) proteins. Lysates from PBS- or brimonidine-treated retinas were analyzed 1, 2, 7, and 14 days after ONI. Representative blots are shown (upper panels). The signal intensity was quantified by densitometry and normalized to β -actin levels. The relative TrkB or p75 protein levels are shown in the graphs. $N = 5–6$. (f) The phosphorylation level of TrkB was determined by immunoblotting of the precipitated TrkB. $N = 5$. * $P < 0.05$, ** $P < 0.01$; one-way factorial ANOVA followed by Scheffé’s multiple comparison test

Discussion

In this study, we demonstrated the following mechanisms underlying the optic nerve regeneration induced by BMD. First, BMD promoted optic nerve regeneration dependent on the activation of Erk1/2. BMD treatment increased the phosphorylation of Erk but not of Akt after ONI. The inhibition of Erk abrogated the optic nerve regeneration induced by BMD. Second, BMD upregulated the retinal expression of TrkB and downregulated the expression of p75. However, inhibition of TrkB did not suppress the optic nerve regeneration induced by BMD.

BMD has been shown to prevent RGC death in various injury models, including glaucoma.¹² It is possible that this effect of BMD might contribute to the promotion of optic nerve regeneration. However, it has been shown that deletion of p53 increases RGC survival, but does not induce axonal regeneration.²² These results indicate that RGC survival is not sufficient to enhance axonal regeneration. Thus, the effect of BMD on the injured axons of the optic nerve may not be dependent on its survival-promoting effect.

It has been shown that alpha2-adrenergic agonists, including BMD, affect the expression of growth factors, neurotrophins, and their receptors.^{13,20,21} BMD did not always upregulate the expression of *bdnf* in our ONI model. This may

be explained by different animal-inherent conditions (e.g., differences between rats and mice and different methods of injury). In this study, it was not clear how BMD induced TrkB and reduced p75 expression after ONI. The precise mechanisms underlying the regulation of gene expression by BMD will be determined in future studies.

Our findings suggest that BMD enhances the neuroprotective signaling pathway and suppresses the axonal growth inhibitory signaling pathway. Several signaling pathways have been associated with Trk receptors, including PI3K, Akt, and MAPK.^{17,18,23} These pathways mediate neuroprotective effects, such as survival, differentiation, and axonal growth. Here, we showed that treatment with BMD increased the expression and phosphorylation of TrkB (Figures 2a and 3b). This may be the reason, at least partly, for the optic nerve regeneration induced by BMD. Conversely, it is known that p75 is required for the axonal growth inhibition mediated by NgR, the receptor for myelin-associated proteins.²⁴ We reported previously that the activation of TrkB and deletion of p75 promote optic nerve regeneration. These findings indicate that p75 opposes the function of TrkB in optic nerve crush injury models. We found that inhibition of Trk by K252a did not inhibit the regrowth-promoting effect of BMD (Figures 1c, 3c and 3d). Therefore, the effect of BMD may

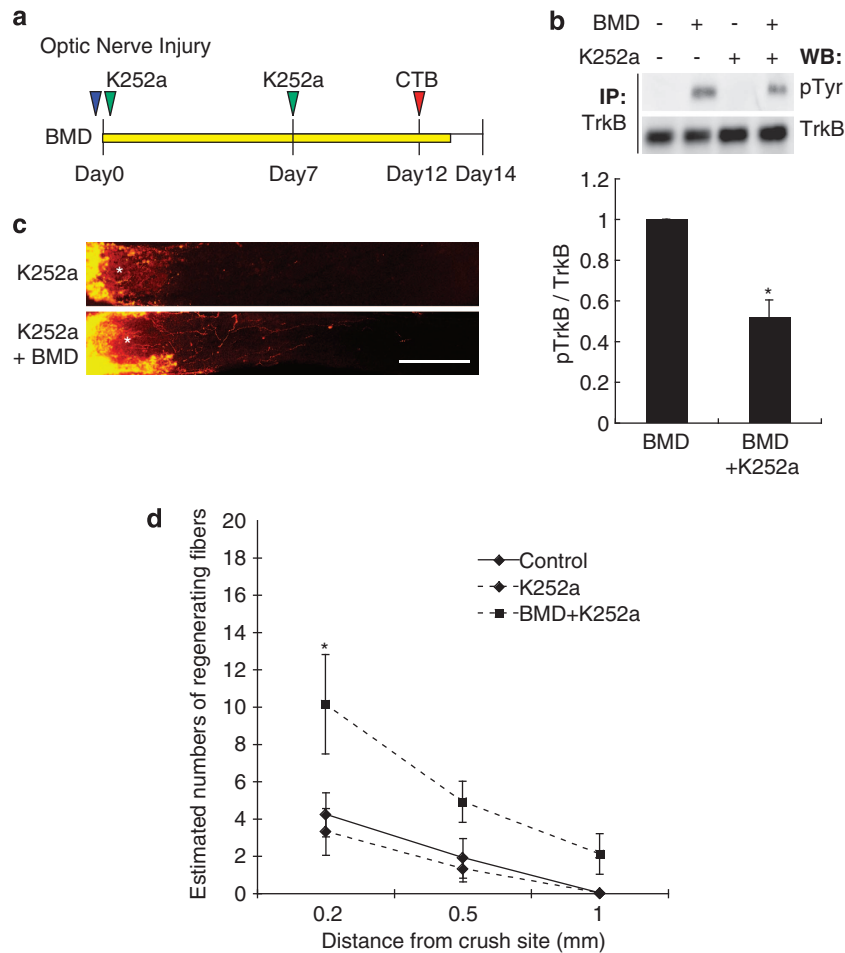


Figure 3 Brimonidine promotes optic nerve regeneration after the application of a Trk inhibitor. **(a)** Schematic representation of the experimental protocol used for intravitreal injection and optic nerve injury. **(b)** Western blot analyses showing the phosphorylation levels of TrkB in retinal extracts prepared from injured mice treated with brimonidine with or without a pan Trk inhibitor, K252a. Anti-TrkB antibodies were used to immunoprecipitate TrkB, and the level of TrkB phosphorylation was determined using anti-phospho-Tyr antibodies. $N = 3$. $*P < 0.05$; Welch's t -test. **(c)** Longitudinal sections through the optic nerve showing CTB-labeled axons distal to the injury site in K252a-treated mice in the presence or absence of brimonidine. *Injury site. Scale bar, 200 μm . **(d)** Quantitative analysis of regenerating axons extending 0.2, 0.5, and 1.0 mm from the end of the crush site at 14 days after injury. Significant differences were noted between PBS- and K252a-treated mice in the brimonidine-treated group. $N = 7$ for each group. $*P < 0.05$; one-way ANOVA followed by Bonferroni's multiple comparison test

be independent of Trk activity. In conclusion, we demonstrated the neuroprotective effect of BMD in an experimental animal model of ONI and elucidated the mechanism underlying BMD-induced optic nerve regeneration.

Materials and Methods

Animals. We purchased C57BL/6J mice (age, 3 weeks) from Japan SLC, Inc. (Shizuoka, Japan). These mice were bred and maintained at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. All experimental procedures were approved by the Institutional Committee of Osaka University.

Reagents and antibodies. The following reagents were used: brimonidine tartrate (1.0%) from Senju Pharmaceutical Co., Ltd. (Osaka, Japan); U0126 (10 μM) from Promega (Madison, WI, USA); and K252a (4 $\mu\text{g}/\mu\text{l}$) from Alomone Labs (Jerusalem, Israel).

The following antibodies were used: mouse anti-p44/42 MAPK (1 : 1000), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204; 1 : 1000), rabbit anti-Akt (1 : 1000), rabbit anti-phospho Akt (Ser473; 1 : 1000), and HRP-conjugated anti-mouse and anti-rabbit IgG secondary antibodies from Cell Signaling Technology

(Danvers, MA, USA); mouse anti-phosphotyrosine (4G10, 1 : 1000) from Millipore (Bedford, MA, USA); rabbit anti-TrkB (1 : 1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); biotinylated anti-TrkB (1 : 2500) from R&D Systems (Minneapolis, MN, USA); streptavidin-peroxidase from Roche Applied Science (Indianapolis, IN, USA); and Alexa Fluor-conjugated secondary antibodies from Molecular Probes (Eugene, OR, USA).

Immunoprecipitation. Retinas were washed with ice-cold PBS and lysed on ice in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 10 mM NaF, and 1 mM Na_3VO_4) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), followed by centrifugation at 4 $^{\circ}\text{C}$ at 15 000 r.p.m for 10 min. The supernatants were incubated with anti-TrkB antibodies for 3 h at 4 $^{\circ}\text{C}$. The immune complexes were collected for 1 h at 4 $^{\circ}\text{C}$ using Protein A-Sepharose (GE Healthcare, Chalfont St Giles, England). After washing the beads four times with the lysis buffer, the proteins were eluted by boiling in 25 μl of 2 \times sample buffer for 5 min, and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting.

Western blotting. The lysates were boiled in sample buffer for 5 min. The proteins were separated on SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with

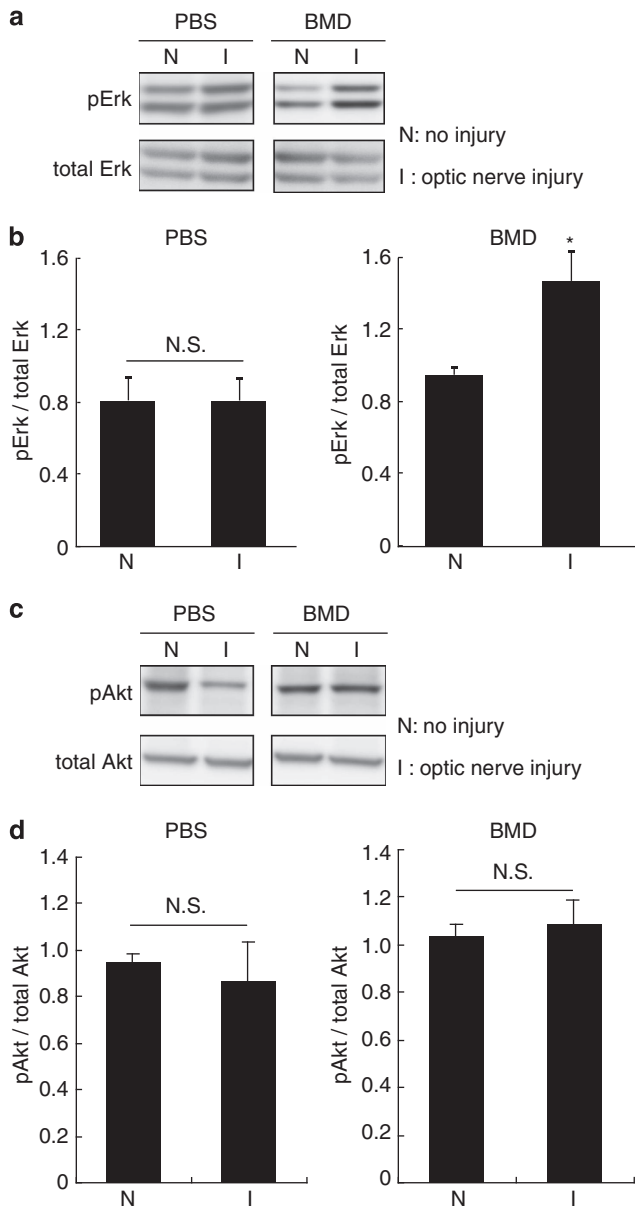


Figure 4 Brimonidine stimulates retinal Erk1/2 activation after optic nerve injury. (a) Western blot analyses showing the phosphorylation levels of Erk1/2 (pErk) in whole retinal homogenates collected 14 days after optic nerve injury from PBS- and brimonidine-treated mice. (b) Graph showing relative phosphorylated Erk1/2 levels compared with the control (intact). (c) Western blot analyses showing the phosphorylation levels of Akt (pAkt), as in a. (d) Graphs showing relative phosphorylated Akt levels compared with the control (intact). The levels of phospho-Akt remained unchanged under all conditions. N: no injury, I: optic nerve injury, $N = 3$ for each group. $*P < 0.05$; Student's *t*-test. NS, not significant

5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) and incubated for 1 h at room temperature or overnight at 4 °C with the primary antibody diluted in PBS-T containing 1% non-fat dry milk. After washing in PBS-T, the membranes were incubated with an HRP-conjugated anti-mouse IgG antibody, an HRP-conjugated anti-rabbit IgG antibody, or streptavidin-peroxidase. For detection, an ECL chemiluminescence system (GE Healthcare) was used. Signals were detected and quantified using the LAS-3000 image analyzer (Fuji Film, Tokyo, Japan).

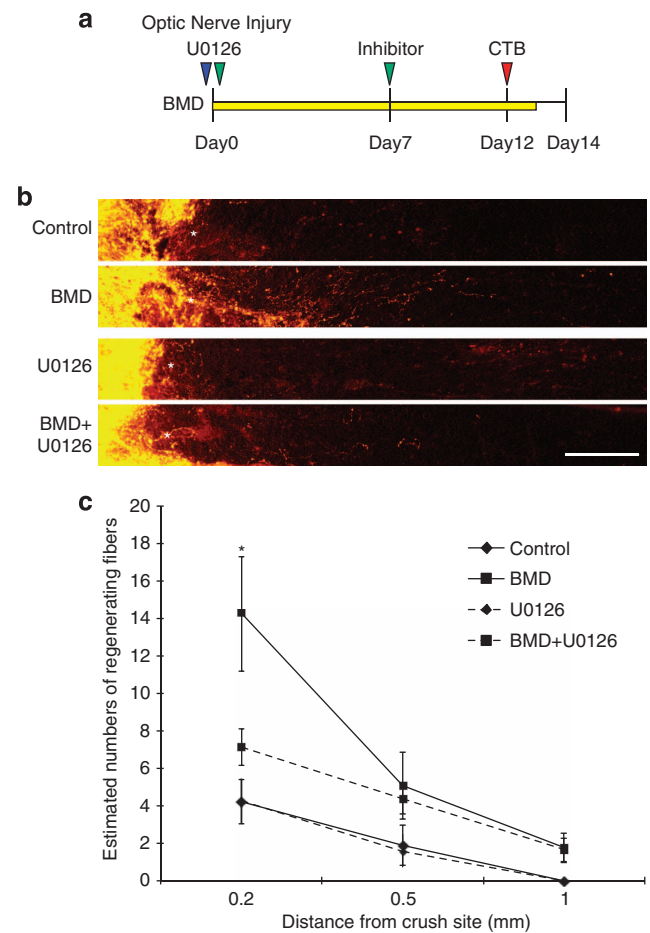


Figure 5 An Erk1/2 antagonist inhibits brimonidine-induced axonal regeneration. (a) Schematic representation of the experiment protocol used for optic nerve injury. Mice were treated with or without brimonidine after the vitreous injection of an Erk1/2 inhibitor, U0126. (b) Longitudinal sections through the optic nerve showing CTB-labeled axons distal to the injury site in mice treated with an Erk inhibitor with or without brimonidine, as in a. *Injury site. Scale bar, 200 μ m. (c) Quantitative analysis of axonal regeneration 14 days after injury. There were no significant differences between the control and U0126-treated groups. $N = 7$ for each group. $*P < 0.05$; one-way factorial ANOVA followed by Scheffé's multiple comparison test

RNA extraction, reverse transcription, and real-time PCR. Total RNA was extracted from retinas using the TRIzol reagent (Invitrogen-Life Technologies, Inc., Carlsbad, CA, USA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems-Life Technologies, Inc., Foster City, CA, USA). The expression of mRNA was examined by real-time PCR using a 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). SYBR green assays were used to quantitate the indicated genes using the primers listed in Table 1, which were prepared as described previously^{25,26,27} and were designed using the Primer Express software (Applied Biosystems). The specificity of each primer set was determined via a pretest that showed the specific amplification of a specific gene by gel visualization and sequencing. A sample volume of 20 μ l was used for SYBR green assays, which contained a 1 \times final concentration of Power SYBR green PCR master mix (Applied Biosystems), 400 nM gene-specific primers, and 1 μ l of template. The PCR cycles started with a uracil-*N*-glycosylase digestion step at 50 °C for 2 min, and an initial denaturation period at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, an annealing phase at 60 °C for 1 min, and a gradual increase in temperature from 60 to 95 °C during the dissociation stage.

Table 1 Sequences of the primer pairs used in real-time PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>Trkb</i>	AAGGACTTTTCATCGGGAAGCTG	TCGCCCTCCACACAGACAC
<i>Bdnf</i>	AGATAAAAAGACTGCAGTGGACATGT	GGATACCGGGACTTTCTCTAGGA
<i>p75</i>	GCAGCTCCCAGCCTGTAGTG	TAGGCCACAAGGCCACAAC
<i>Gapdh1</i>	AATGTGTCCGTCGTGGATCTG	CAACCTGGTCCCTCAGTGTAGC
<i>Gapdh2</i>	GGGCTGGCATTGCTCTCA	TGTAGCCCGTATTTCATTGTCATACCA
<i>Gapdh3</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTCT

Gapdh1 for *Trkb*; Gapdh2 for *Bdnf*; Gapdh3 for *p75*

The relative mRNA expression was normalized by measurement of the amount of *Gapdh* mRNA in each sample. The results of cycle threshold values (Ct values) were calculated using the $\Delta\Delta C_t$ method, to obtain fold differences.

ONI and anterograde labeling. ONI was performed as described previously.¹⁶ Briefly, the left optic nerves of postnatal day 21 mice were exposed intraorbitally and crushed with fine forceps. The mice were given drops of 1% brimonidine tartrate daily for 2 weeks. On day 12 after axotomy, 2 μ g of Alexa 488 or 555-conjugated CTB (Invitrogen) was injected into the vitreous body using a glass needle. On day 14 after axotomy, the animals were perfused with 4% paraformaldehyde and the lens and vitreous body were removed. The remaining eye cup, which contained the nerve segment, was postfixed and immersed in 10–30% sucrose overnight at 4 °C. The eye cups were then embedded in OCT compound (Tissue Tek; Sakura Finetek, Torrance, CA, USA). Tissues were frozen in dry ice, and 16 μ m serial cross-sections were prepared using a cryostat and collected on MAS-coated glass slides (Matsunami Glass, Osaka, Japan).

Intravitreal injection. After ONI, intravitreal injections were performed using a 5- μ l Hamilton syringe with a glass needle. The eye was punctured at the upper nasal limbus, and 1 μ l of reagent solution (4 μ g/ μ l K252a or 10 μ M U0126) or control vehicle was injected in the eye. Because reflux of a certain amount of intraocular fluid is unavoidable when the needle is removed from the injection site, the glass needle was kept in place for 10 s, to allow the diffusion of the solution. Repeat injections were performed on day 7 after axotomy.

Quantification of axonal regeneration. Axonal regeneration was quantified by counting the number of CTB-labeled axons extending 0.2, 0.5, and 1.0 mm from the end of the crush site in four different sections. The cross-sectional width of the nerve was measured at the point where the counts were taken, and used to calculate the number of axons per ml of nerve width. This number was then averaged over the five sections. The total number of axons extending a distance d in a nerve with a radius r ($\sum ad$) was estimated by summing the number in all the sections with a thickness t (16 μ m), using the following formula.

$$\sum ad = \pi r^2 \times [\text{average axons/mm}] / t$$

Statistical analysis. The data are presented as the mean \pm S.E.M. of at least three independent experiments. Statistical analyses were performed using Student's *t*-test (Figures 1c, 4b, and 4d), Welch's *t*-test (Figure 3b), one-way ANOVA followed by Scheffé's test (Figures 2a–f, and 5c), and Bonferroni's multiple comparison test (Figure 3d). *P*-values < 0.05 were considered significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Agency (JST), and by a grant-in-aid for young scientists from the Japan Society for the Promotion of Science.

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