Research report

Neuroprotective effects of brain-derived neurotrophic factor in eyes with NMDA-induced neuronal death

Noriaki Kido\textsuperscript{a}, Hidenobu Tanihara\textsuperscript{b,*,} Megumi Honjo\textsuperscript{a}, Masaru Inatani\textsuperscript{a}, Tohru Tatsuno\textsuperscript{c}, Chikao Nakayama\textsuperscript{c}, Yoshihito Honda\textsuperscript{a}

\textsuperscript{a}Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan
\textsuperscript{b}Department of Ophthalmology, Tenri Hospital, Mishima-cho 200, Nara 632-8552, Japan
\textsuperscript{c}Sumitomo Pharmaceuticals Research Center, Osaka, Japan

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Abstract

\textbf{Purpose:} To determine if brain-derived neurotrophic factor (BDNF) has a neuroprotective effect against \textit{N}-methyl-\textit{D}-aspartate (NMDA)-induced cell death in retina. \textbf{Methods:} NMDA was injected into the vitreous of rat eyes. NMDA-induced neuronal death was measured by morphometric analyses on cell counts of ganglion cell layer cells and thickness of retinal layers. Also, we conducted additional experiment using retrograde labeling with a fluorescent tracer (Fluoro-Gold) for exact counting of retinal ganglion cells (RGCs). In addition, intravitreal glutamate levels were measured with the use of a high-performance liquid chromatography (HPLC) system. \textbf{Results:} Morphometric analysis of retinal damage in NMDA-injected eyes showed that BDNF could protect inner retinal cells from glutamate receptor-mediated neuronal death. Also, counts of RGCs labeled with a fluorescent tracer showed that BDNF could protect RGCs from glutamate receptor-mediated neuronal death. Furthermore, measurements of intravitreal glutamate levels indicated an increase in this excitatory amino acid in the vitreous after NMDA injection. \textbf{Conclusions:} Exogenous BDNF can protect inner retinal cells (possible RGCs and amacrine cells) from NMDA-induced neuronal death. However, increased intravitreal glutamate levels in response to NMDA-mediated neurotoxicity may augment retinal degeneration. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

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1. Introduction

Several growth factors and neurotrophic agents are important for regulation of neuronal development and maintenance of the adult central and peripheral nervous systems [15]. Brain-derived neurotrophic factor (BDNF) supports survival of retinal ganglion cells (RGCs) [9,10,16,20,30] and has been shown to act as a target-derived trophic factor [16]. Thus, it is likely that damage to neural axons in the optic nerve may cause inadequacy of target-derived trophic factors, and result in RGCs death. In the pathogenesis of glaucoma and other optic neuropathies, this mechanism is hypothesized to at least partially explain why damage to neurons of the optic nerve results in death of RGCs. On the other hand, some investigations showed that RGCs receive BDNF mainly from intraretinal sources [5] and retrograde BDNF cannot rescue RGCs in the long term [8]. Thus, although further studies are needed for exact identification of intraocular origin and physiologic role for BDNF, it is hypothesized to play important roles in the development and maintenance of retinal tissue.

The presence of TrkB, a high affinity BDNF receptor, also implies a potential of BDNF as a neuroprotective factor against numerous ocular diseases such as glaucoma, ischemic retinal diseases and retinal degenerative diseases [2]. In a number of animal models of ocular diseases, BDNF has been reported to protect retinal neuronal cells from injuries such as light exposure [12], ischemia [28],...
retinal degeneration [13] and axotomy [10,19,26,30]. Furthermore, BDNF is involved in regeneration of axotomized retinal neurons [17,26] and supports survival of RGCs in culture [9].

The glutamate receptor-mediated neurotoxicity is regarded as one important mechanism in the pathogenesis of neurodegenerative diseases [11,14,18,23,27,29]. Indeed, in a number of ocular diseases such as glaucoma [4], ischemia [3,22,24] and optic neuropathy [32], elevated levels of glutamate have been reported. Thus, elevated glutamate concentrations are hypothesized to be a common pathway of visual threatening diseases and to be involved in augmentation of neurodegenerative processes. As mentioned above, although BDNF has been regarded to possibly be a neuroprotective drug against many ocular diseases [1], knowledge about its neuroprotective mechanism in animal models of retinal damage is limited [13,28,30,31]. In particular, there are no conclusive data about whether BDNF inhibits primary (and disease-specific) damaging processes in neuronal lesions, or secondary (and probably more extensive) neurodegenerative events. Among the known mediators involved in secondary neurodegenerative processes, abnormal release and/or uptake of glutamate and resultant stimulation of some glutamate receptors have been regarded as central events in many neurodegenerative processes [3,4,22,24,32]. N-methyl-D-aspartate (NMDA) glutamate receptors are known to be involved in neuronal cell death, including retinal neuronal cell death [11,14,18,27]. Herein, we report that BDNF protects retinal neuronal cells from glutamate receptor-mediated neurodegenerative damage.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague Dawley rats (6 weeks after birth) which were housed at room temperature on a 12-h light/12-h dark cycle and which were given water and food ad libitum. The animals were killed by intraperitoneal overdose injection of pentobarbitral, and the eyes were enucleated. Eyes were immersed in fixative solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C, followed by dehydration and paraffin embedding. Transverse sections with a thickness of 3 μm were made of rat retinas through the optic disc. The sections were stained with hematoxylin and eosin and subjected to morphometric analysis. The degree of NMDA-induced retinal neuronal death was quantified by cell counts in the ganglion cell layer (GCL) and by the thickness of the inner plexiform layer (IPL) at 1.0 to 1.5 mm from the optic disc. Data from the three sections were averaged for each eye.

2.4. Analysis for the number of fluoro-gold labeled RGCs

Retrograde labeling was made in a manner similar to that described by Sawada et al. [25]. Briefly, rats were anesthetized with xylazine and ketamine and then the heads were fixed in a stereotaxic apparatus. Fluoro-Gold (Fluorochrome, Englewood, CO, USA) was microinjected bilaterally into the superior colliculi of rats. Three days after Fluoro-Gold injection, the animals were killed by intraperitoneal overdose injection of pentobarbitral and the eyes were enucleated. Eyes were fixed with 4% paraformaldehyde. Retinas were divided into six radial cuts and removed from the sclera and mounted on slides. Analysis for the number of Fluoro-Gold labeled RGCs was carried out. Briefly, the regions for counting the number of RGCs were selected from two fields of the central area (1 mm from the optic disc) and two fields of the peripheral area (4 mm from the optic disc) on each retinal cut from six radial cuts. Thus, in each eye, a total of 24 fields (12 fields from the central areas and 12 fields from the peripheral areas) were examined for counting the labeled RGCs.

2.5. Amino acid analysis

At 0, 1, 3, 7 and 14 days following intravitreal injection of NMDA, the vitreous humor was collected by cutting the eye and the vitreous acidified with one-tenth volume of 4 N perchloric acid. Before amino acid analysis, the medium was neutralized with 2.5 M potassium carbonate, spun at 10,000×g for 10 min at 4°C [33] and the supernatant fraction analyzed by a micro high-performance liquid chromatography (HPLC) system for automated analysis of
3. Results

3.1. Morphometric analysis for neuroprotective effects of BDNF

In eyes that have undergone intravitreal injection of NMDA (200 nmol), significant cell loss in the GCL and thinning of IPL were observed after 7 days, as described previously [6]. In an effort to elucidate the neuroprotective effects of BDNF against NMDA-mediated retinal damage, we conducted morphometric analyses. At 2 days after injection of 1 μl of BDNF (0.1, 1 or 10 μg) into the vitreous, 200 nmol of NMDA was injected into the same animals. Phosphate-buffered saline (PBS) was injected instead of BDNF-containing solution in some animals as a negative control (Fig. 1). In NMDA (200 nmol)-injected eyes, the mean numbers (±standard error) of GCL cells were 25.9±0.9, 33.6±1.5, 36.9±2.0 and 37.8±5.4, in PBS-injected eyes, 0.1 μg BDNF-injected eyes, 1 μg BDNF-injected eyes and 10 μg BDNF-injected eyes, respectively. Statistical analysis showed that intravitreal injection of BDNF of 1 and 10 μg has significant protective effects against NMDA-induced retinal damage [PBS vs. BDNF (0.1 μg), P=0.0788; PBS vs. BDNF (1 μg), P=0.0030, PBS vs. BDNF (10 μg), P=0.0017, ANOVA] (Fig. 2). The mean thickness (±standard error) of the IPL at 0, 3 and 7 days after injection of NMDA (200 nmol) were 36.2±1.6, 23.9±1.8 and 23.9±1.8 μm, respectively. The differences between the thickness of the IPL in PBS- and BDNF-injected eyes was statistically significant on the seventh post-treatment day (P=0.0135, ANOVA), but not on day 0 (P=0.5651) and day 3 (P=0.3860) (Fig. 4).

After pretreatments with intravitreal injection of BDNF, the mean thickness (±standard error) of the IPL at 0, 3 and 7 days after injection of NMDA (200 nmol) was 36.2±1.6, 22.2±1.5 and 24.0±1.8 μm, respectively. In PBS-injected (control) eyes, the mean thickness (±standard error) of the IPL at 0, 3 and 7 days after injection of NMDA (200 nmol) was 39.2±1.0, 21.3±2.6 and 18.8±1.4 μm, respectively. The difference between the thickness of the IPL in PBS- and BDNF-injected eyes was statistically significant on the seventh post-treatment day (P=0.0135, ANOVA), but not on day 0 (P=0.1691) or day 3 (P=0.8371) (Fig. 5).

3.2. Analysis of neuroprotective effects of BDNF in experiments using retrograde labeling of RGCs

In an effort to investigate if BDNF can actually protect RGCs from NMDA-induced neuronal death, we conducted retrograde labeling of RGCs with Fluoro-Gold (Fig. 6). At first, in PBS-injected eyes without NMDA (200 nmol), the mean density (±standard error) of RGCs was 2342.3±103.4/mm². Then, 2 days after injection of BDNF (1 μg) (or PBS in control experiments) into the vitreous, 200 nmol of NMDA was injected in the same animals. At 7 days after NMDA-injected eyes, in control (PBS-injected) and experimental (BDNF 1 μg-pretreated) eyes, the mean density (±standard error) of RGCs was 269.9±24.2 and 561.1±19.8/mm², respectively, which shows a statistically significant difference (P=0.0092, ANOVA) (Fig. 7).

GCL
IPL
INL
OPL
ONL

PBS
PBS + NMADA (200 nmol)
BDNF (0.1 μg) + NMADA (200 nmol)
BDNF (1 μg) + NMADA (200 nmol)
BDNF (10 μg) + NMADA (200 nmol)

Fig. 1. Light microscopic photographs of representative retinal tissues in control and experimental eyes at 7 days after intravitreal injection of NMDA (200 nmol).
Fig. 2. Morphometric analysis of protective effects of BDNF in NMDA-injected eyes. In NMDA (200 nmol)-injected eyes, counts (cell number per mm) of cells present in the ganglion cell layer (GCL) were evaluated. Data from three sections were averaged for each eye. The results are shown as the mean±standard error. Morphometric analysis showed statistically significant protective effects of BDNF (1 and 10 μg) to the decrease in GCL cells (P<0.0030 and P<0.0017, ANOVA). NS indicates not significant.

Fig. 3. Morphometric analysis of protective effects of BDNF in NMDA-injected eyes. In NMDA (200 nmol)-injected eyes, the thickness (μm) of the inner plexiform layer (IPL) was evaluated. Data from three sections were averaged for each eye. The results are shown as the mean±standard error. Morphometric analysis showed protective effects of BDNF (1 and 10 μg) to the decrease in IPL thickness. NS indicates not significant.
Fig. 4. Time course of morphological changes in protective effects of BDNF on NMDA-induced retinal toxicity. With the pretreatments with intravitreal injection of BDNF, counts (cell number per mm) of cells present in the ganglion cell layer (GCL) at 0, 3 and 7 days after NMDA (200 nmol) injection were evaluated. Data from three sections were averaged for each eye. The results are shown as the mean±standard error. Morphometric analysis showed statistically significant protective effects of 7 days after NMDA injection to the decrease in GCL cells ($P<0.0001$, ANOVA). NS indicates not significant.

Fig. 5. Time course of morphological changes in protective effects of BDNF on NMDA-induced retinal toxicity. With the pretreatments with intravitreal injection of BDNF, the thickness (μm) of the inner plexiform layer (IPL) at 0, 3 and 7 days after the NMDA (200 nmol) injection was evaluated. Data from three sections were averaged for each eye. The results are shown as the mean±standard error. Morphometric analysis showed statistically significant protective effects of 7 days after NMDA injection to the decrease in IPL thickness ($P=0.0135$, ANOVA). NS indicates not significant.
3.3. Amino acid analysis of vitreous humor

With the use of HPLC, concentrations of glutamate in vitreous humor from experimental (BDNF-injected) and control (PBS-injected) eyes were measured. As shown in Fig. 8, the mean (±standard error) concentrations of glutamate in vitreous humor of PBS-injected eyes at 0, 1, 3, 7 and 14 days after the NMDA injection were 166.14±23.04 (n=9), 127.00±15.16 (n=9), 120.35±18.67 (n=6), 180.93±27.37 (n=6) and 280.80±33.10 (n=7) pmol, respectively. Statistical analysis showed a significant increase in glutamate concentrations at the 14 days after treatment day in comparison with day 0 (P=0.0055, ANOVA). The mean (±standard error) concentration of glutamate in vitreous humor of eyes pretreated with BDNF (1 μg) at 0, 1, 3, 7 and 14 days after the NMDA injection was 126.20±3.77 (n=4), 115.93±34.11 (n=4), 84.48±16.40 (n=4), 263.06±40.45 (n=5) and 347.40±161.76 (n=3) pmol, respectively. There were no statistically significant differences between glutamate concentrations in PBS- and BDNF (1 μg)-treated eyes (P=0.4039, P=0.8165, P=0.4849, P=0.0913 and P=0.2269, respectively, at day 0, 1, 3, 7 and 14, respectively, ANOVA). Furthermore, the mean (±standard error) concentrations of glutamate in vitreous humor of eyes pretreated with BDNF (10 μg) at 0, 1, 3, 7 and 14 days after the NMDA injection were 127.50±29.77 (n=5), 115.48±40.92 (n=4), 141.20±31.39 (n=4), 71.80±12.87 (n=4) and 109.60±12.06 (n=4) pmol, respectively. There were significant differences between glutamate concentrations in PBS- and BDNF (10 μg)-treated eyes at days 7 and 14 after the NMDA injection (P=0.0364 and P=0.0010, ANOVA) (Fig. 8).

4. Discussion

Several neurotrophic factors have been reported to promote the survival of axotomized and naturally dying retinal neural cells [9,16,19,26,30]. BDNF, ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) have been shown to have axon-maintaining effects on damaged optic nerve fibers and to promote survival of RGCs, amacrine cells and photoreceptors in diseased retinas [2,12,31]. Among them, BDNF has been reported to protect retinal neuronal cells from various injuries, including light exposure [12], ischemia [28], retinal degeneration [13] and axotomy [19,26,30]. In addition, because BDNF
Fig. 7. Analysis of neuroprotective effects of BDNF on labeled RGCs after NMDA injection. At 7 days after the NMDA-injection, in NMDA (200 nmol)-injected eyes, counts (cell number per mm²) of the labeled cells were counted. Data from the 24 fields in two (central and peripheral) regional areas were added for each eye. The results are shown as the mean ± standard error. Statistical analysis showed a statistically significant difference between the cell counts in control and experimental [BDNF (1 μg)-pretreated] eyes (P=0.0092, ANOVA). NS indicates not significant.

Fig. 8. Time course of glutamate concentrations in the vitreous humor of experimental eyes. At 7 and 14 days after injection of NMDA, glutamate concentrations in vitreous humor increased significantly. Pretreatment with BDNF (10 μg) had a significant inhibitory effect on the increase in glutamate levels in vitreous humor of experimental eyes.
acts as a target-derived trophic factor [16], damage in neural axons and resultant inadequacy of retrograde supply of this neurotrophic factor may play a part in the neurodegenerative processes seen in glaucoma and other optic neuropathies. This is why BDNF is regarded as a potential neuroprotective drug against any ocular conditions, such as glaucoma, ischemia, traumatic injury and retinal degenerative diseases. In these diseases, elevated levels of glutamate in the vitreous have been reported [3,4,22,24,32], so it is quite possible that abnormal levels of glutamate result in more extensive and serious secondary damage in neural tissues. However, to our knowledge, there were no reports about the selective neuroprotective effects of BDNF against glutamate receptor-mediated secondary neurodegeneration in retina. Our attempt is to examine the possibility that BDNF protects against retinal neuronal damage from secondary neurodegenerative processes.

Our morphologic studies demonstrated that injection of NMDA, a ligand for glutamate receptors related to neuronal cell death results in statistically significant cell loss in the GCL and thinning of the IPL as described elsewhere [6], which is in agreement with a previous report [21]. In our previous study [6], in NMDA-injected eyes, up-regulated expression of another neurotrophic factor, CNTF, has been found in the retina. Our morphometric studies showed the neuroprotective effects of CNTF against NMDA-induced retinal damages. Also, neuroprotective effects of BDNF against the retinal neurotoxicity mediated by NMDA have been shown by our results in the present study. In the present study, we have conducted a series of experiments in an attempt to elucidate protective effects of BDNF in NMDA-related retinal damage. Our morphometric analysis showed that intravitreal injection of BDNF (1 μg) results in less cell loss in the GCL and less thinning of the IPL, but injection of BDNF (0.1 μg) does not. Interestingly, intravitreal injection of a larger amount (10 μg) of BDNF does not seem to have a significant additive neuroprotective effect. Furthermore, an additional experiment using retrograde labeling of RGCs with Fluoro-Gold showed that BDNF protects RGCs in the GCL from glutamate receptor-mediated neuronal death. Statistically significant protective effects of BDNF are seen at 7 days after the NMDA treatment.

In the present study, intravitreal injection of BDNF was done in an attempt to obtain a neuroprotective effect against glutamate receptor-mediated retinal damage. The injected BDNF can bind to and be sustained in retinal tissues. Also, our previous studies showed that, in animal experiments with chemically induced retinal hypoxia, BDNF contains more protective effects on retinal damages when it was administered 2 days before the onset of retinal hypoxia compared to 1 h before [7]. Although we were unable to identify the exact mechanisms of this phenomenon, induction of some substances related to the protective effects of BDNF has been hypothesized. From our experience in the previous study [7], BDNF was administered intravitreally 2 days before NMDA injection in the present study. In addition, intravitreal injection of BDNF (10 μg) results in inhibition in elevations of glutamate concentrations in the vitreous of eyes with retinal damage including NMDA-mediated neurotoxicity. Similar findings were previously obtained from the measurements of intravitreal glutamate levels in eyes with chemically-induced retinal ischemia [7]. So, despite these limitations of BDNF as a candidate for a possible neuroprotective drug for ocular diseases, inhibition of release and/or uptake in diseased retinas suggests an inhibitory effect against secondary neurodegenerative events, which are common in many retinal diseases.

BDNF has been regarded as a target-derived trophic factor [16]. However, some investigators have shown that RGCs receive BDNF mainly from intraretinal sources [5]. In addition, recently, Isenmann et al. have reported that retrograde BDNF cannot rescue RGCs on the long term [8]. Thus, these findings suggest that the retrograde degeneration of RGCs after lesion of the optic nerve may be the results of the physical lesion inflicted to the nerve instead of deprivation from target-derived neurotrophic factors. Also, since intravitreal injection of BDNF is effective for survival of RGCs in our present study, intravitreal or intraretinal BDNF may be essential for RGCs rather than superior colliculi-derived BDNF.

In conclusion, we suggest that exogenous BDNF can protect retinal neuronal cells in the inner retinal layers from glutamate receptor-mediated neuronal death, and that this neurotrophic factor can be potentially a clinically useful drug for many retinal diseases.

Acknowledgements


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