Neuroprotective Effects of C-Type Natriuretic Peptide on Rat Retinal Ganglion Cells

Jia Ma, Wenban Yu, Yun Wang, Guiquin Cao, Suping Cat, Xiaoming Chen, Naibong Yan, Yuansheng Yuan, Hong Zeng, Debra L. Fleenor, Xuyang Liu, and Iok-Hou Pang

PURPOSE. To evaluate the potential neuroprotective effects of C-type natriuretic peptide (CNP) on rat retinal ganglion cells (RGCs).

METHODS. Cultured adult rat retinal cells were treated with vehicle, CNP, or atrial natriuretic peptide (ANP), followed by cytotoxic insults (glutamate, TNFα, or withdrawal of trophic factor). RGC survival was analyzed by counting Thy-1-positive cells in each well. For in vivo evaluation, N-methyl-D-aspartate (NMDA) with or without CNP was injected intravitreally into rat eyes. At various time points after injection, retinal cross-sections were analyzed. Thickness of the inner nuclear layer, and retinal flat mounts were assessed by counting cresyl violet-labeled or TUNEL-positive cells. Expressions of natriuretic peptide receptor-B (NPRB) and apoptosis-related genes in retina, including Bcl-xL, BAX, and μ-calpain, were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

RESULTS. At 50 and 500 nM, CNP, but not ANP, significantly (P < 0.05) protected against glutamate-insult and trophic factor withdrawal-induced RGC death in vitro. Neither peptide significantly affected TNFα-induced cytotoxicity. Intravitreal injection of NMDA (20 nanomoles) significantly (P < 0.05) decreased the thickness of the inner plexiform layer (IPL), induced cell loss, increased the number of TUNEL-positive cells in the RGC layer, and upregulated the expression of Bcl-xL, BAX, and μ-calpain. All these effects were significantly (P < 0.05) alleviated by concomitant injection of CNP (4.5 nmol, 10 μg). The neuroprotective effects of CNP were maintained up to 14 days after CNP injection.

CONCLUSIONS. CNP protects rat RGCs against the apoptotic damage induced by insults such as excitatory amino acid, both in vitro and in vivo. (Invest Ophthalmol Vis Sci. 2010;51: 3544-3553) DOI:10.1167/iovs.09-5049

From the 1Ophthalmic Laboratories and Department of Ophthalmology, West China Hospital, Sichuan University, Chengdu, People’s Republic of China; the 2Department of Ophthalmology, the First Affiliated Hospital, Kunming Medical College, Kunming, People’s Republic of China; the 3Department of Ophthalmology, West China Hospital, Sichuan University, Chengdu, People’s Republic of China; and the 4Glaucoma Research, Alcon Research, Ltd., Fort Worth, Texas.

*These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by NNSF (National Natural Science Foundation of China) Grants 30872830 and 30872839, and the National High-tech R&D Program of China, 863 Program, 2006AA02A117.

Submitted for publication December 10, 2009; revised February 4, 2010; accepted February 4, 2010.

Disclosure: J. Ma, None; W. Yu, None; Y. Wang, None; G. Cao, None; S. Cai, None; X. Chen, None; X. Yan, None; Y. Yuan, None; H. Zeng, None; D.L. Fleenor, None; X. Liu, None; I.-H. Pang, None

Corresponding author: Xuyang Liu, Ophthalmic Laboratories and Department of Ophthalmology, West China Hospital, Sichuan University, Chengdu, 610041, People’s Republic of China; xliu1213@gmail.com.

Glaucoma is a leading cause of vision loss worldwide. It is estimated that 60.5 million people will have glaucoma in 2010, increasing to 79.6 million by 2020. Glaucoma is a progressive optic neuropathy and retinopathy, caused by intraocular pressure (IOP) and other possible risk factors, such as age, race, family history, diabetes, pathologic myopia, and so on, resulting in the loss of retinal ganglion cells (RGCs) and eventually causing irreversible functional visual defect. Although elevated IOP, among all risk factors, is an important one, reduction of IOP alone is often insufficient to prevent progression of glaucoma in some patients. Therefore, neuroprotective strategies to minimize or delay RGC loss are critical for the future direction of glaucoma therapy.

The pathophysiology mechanism underlying RGC death in glaucoma remains unclear. Several theories have been proposed—for example, excitotoxicity, deprivation of trophic factors, vascular insufficiency, reactive gliosis, and immunologic abnormality. However, none has been universally accepted. Correspondingly, various neuroprotective compounds and methods have been evaluated. Some are specifically designed to address a particular mechanism, whereas others can protect RGCs against multiple insults.

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family. Other members include atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). These peptides interact with cytoplasmic membrane-spanning receptors, designated natriuretic peptide receptor (NPR)-A and -B. NPR-A has higher affinities for ANP and BNP, whereas NPRB preferentially binds to CNP. The receptors themselves are guanylyl cyclases. When activated, they exert their biological effects, mainly by increasing the production of cyclic (c)GMP. Systemically, the peptides are involved in diuresis, natriuresis, vasorelaxation, and antimitogenesis.

In the eye, NPRs have been found in the anterior segment, such as ciliary processes, ciliary muscle, and trabecular meshwork. Activation of these receptors increases the cGMP level in the cell, affects calcium influx, and in the case of ciliary muscle, decreases contractility. Functionally, the natriuretic peptides are potent ocular hypotensives. Intraocular injection of ANP, BNP, or CNP can lower rabbit IOP for hours or even days. Among these peptides, CNP is the most potent IOP-lowering member, indicating that NPRB is the major receptor subtype that mediates this effect.

Natriuretic peptides and NPRs have also been found in the retina. By immunohistochemistry, ANP, BNP, and CNP were detected in the inner and outer plexiform and nuclear layers of the human retina, as well as the cytoplasm of RGCs. In the rat and rabbit, the distribution of these peptides is similar, except that ANP appears absent in the RGCs. Interestingly, ANP, BNP, and CNP were located in different parts of rat Müller cells. ANP and BNP are present in the main trunks and major processes, whereas, CNP localizes in the endfeet and major processes. These findings raise a possibility that these peptides, when released from the Müller cells, perform layer-
specific functions in the retina.27 Expression of NPR-A and -B in human and rat retina was verified by molecular cloning and RT-PCR.18,26,29–31 NPR-A expression in the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer in the rat retina has been shown by immunohistochemistry.52 Although their biological functions in the retina are not well understood, the presence of natriuretic peptides and their receptors suggests a potential autocrine or paracrine function. Analogous to their putative roles in other tissues, the natriuretic peptides may modulate ion and fluid distributions between the intra- and extracellular space in the retina. They may serve as neurotransmitters or neuromodulators, similar to other neuropeptides. Most important, they may even act as neuroprotectants. In fact, ANP was implicated as contributing to the spreading-depression-induced neuroprotection in the rat cerebral cortex.53 In cultured PC12 cells, both ANP and BNP inhibit apoptotic DNA fragmentation and increase cell survival.54 Recently, ANP was demonstrated to ameliorate NMDA-induced retinal neurotoxicity via NPR-A in the rat retina. Its effect is most likely mediated by dopamine and activation of the dopamine D1 receptor.52 To evaluate the potential direct neuroprotective effects of natriuretic peptides on RGCs, we tested ANP and CNP for their protective effects against several insults in cultured adult rat retina cells. We found that CNP, but not ANP, was efficacious. We then further evaluated CNP in an NMDA model of retinopathy and found that pretreatment with CNP provided a neuroprotective effect against NMDA-induced cell death in the retina.

METHODS

Experimental Animals

Young adult male Sprague-Dawley rats (~8-week-old; body weight, 180–220 g) were used in the studies. They were housed in rooms where temperature (23 ± 1°C), humidity (55% ± 5%), and lighting (12-hour light/12-hour dark cycle) were controlled, and water and food were available ad libitum. The rats used in cultured RGC studies were from Charles River Laboratories (Wilmington, MA), and those used in the in vivo studies were from the Experimental Animal Center of Sichuan University (Chengdu, People’s Republic of China). All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Ganglion Cell Culture

Culture of retinal neurons was performed as reported.35 Briefly, rats were euthanatized by CO2 asphyxiation and their eyes enucleated. The retina from each eye was dissected and incubated in a papain solution, containing 2 mg/mL papain (Sigma-Aldrich, St. Louis, MO), 0.4 mg/mL β-cysteine (Sigma-Aldrich), and 0.4 mg/mL bovine serum albumin (Sigma-Aldrich) in cell growth medium (Neurobasal; Gibco/Invitrogen, Carlsbad, CA), for 25 minutes at 37°C, then washed three times with culture medium. The cells were cultured in this medium with the three trophic factors, bFGF, BDNF, and CNTF, were removed from the culture medium. The cells were cultured in this medium with the indicated compounds for 3 days. At the end of the incubation period, the cells were fixed and labeled for Thy-1, an RGC marker, by immunocytochemistry (primary antibody against Thy-1, diluted 1:500; Chemicon, Temecula, CA; secondary antibody: AlexaFluor 594-labeled goat anti-mouse IgG, 1:300; Invitrogen/Molecular Probes, Eugene, OR). Cell survival was quantified by counting Thy-1-positive cells in each well. For each test condition, three wells were evaluated in each of two or three separate studies (n = 6–9).

Intravitreal Injection

Before the in vivo studies, all eyes were examined routinely to exclude any disorders of the cornea, iris, pupil, lens, vitreous, and retina. On the day of intravitreal injection, the rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (3 mL/kg body weight; Kelong, Chengdu, China) and a topical drop of 0.4% hydrochloric oxybuprocaine (Santen, Osaka, Japan) on the cornea, and their pupils were dilated with 1% tropicamide (Mydri-p; Japan). A single 5-μL bolus of (1) sterile 0.1 M PBS (pH 7.4) as the control, (2) 4 mM NMDA (total injected amount, 20 nanomoles; Sigma-Aldrich) in PBS, (3) 0.9 mM CNP (total injected amount, 4.5 nanomoles or 10 μg) in PBS, or (4) 4 mM NMDA+0.9 mM CNP, was injected intravitreally with a 10-μL microsyringe (Hamilton, Reno, NV) under an ophthalmic surgical microscope. Rats with postsurgical complications such as cataract or intravitreal hemorrhage were excluded from the studies. The remaining rats were killed by an intraperitoneal overdose injection of 10% chloral hydrate at the indicated time points (as shown in Figs. 2–8). The eyes were enucleated and prepared for the following assessments.

Retina Cross-Section

Enucleated eyes were fixed in 4% paraformaldehyde (Kelon, Chengdu, China) in 0.1 M PBS (pH 7.4) for 24 hours at 4°C, followed by dehydration and paraffin embedding. Four-μm-thick cross sections through the optic disc were produced. The sections were dewaxed, rehydrated, and stained with hematoxylin-eosin (HE). Images of each section were acquired at 200× magnification, with a light microscope for morphologic analysis. Thickness measurements of various retinal layers were performed at a region 1.0 to 1.5 mm from the margin of the optic nerve head. Four images of each retina were chosen, and each image was measured three times. The mean of the 12 measurements for each retina was used as a single value for each retina. All quantifications were performed in a masked manner. For each time point of each test condition, four animals were used (n = 4).

Retina Flat Mount

Rat eyes were oriented by a suture placed around the insertion of the superior rectus muscle, enucleated, fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 2 hours at room temperature, and washed two
times in 0.1 M PBS. After the anterior segment was removed, a cut through the posterior eye cup was made opposite to the suture marker toward the optic nerve head. The retina was carefully separated from the sclera and dissected from the eye cup at the optic nerve head. The isolated retina was then placed on a 1% gelatin-treated glass slide with the vitreous side facing up, flattened by three shorter cuts, and flat mounted with a fine brush. The whole-retina flat mount was air dried and then immersed in 0.5% Triton X-100 for 1 minute.

**Ganglion Layer Cell Count**

The treated retina flat mounts were stained with 0.1% cresyl violet for 20 to 30 minutes, dehydrated, and mounted with Canada balsam. Each retina was divided into superior-temporal, inferior-temporal, superior-nasal, and inferior-nasal quadrants. Each quadrant was further divided into central, midcentral, and peripheral regions. An image from each region, equivalent to 4.7 × 10⁻³ mm² area, was acquired via a light microscope (Eclipse TE2000-U, Nikon Corp., Japan) at 400× magnification. The number of cresyl violet-stained neurons in the 12 images was counted; each image was counted three times. All 36 counts were averaged to give a mean value for each retina, expressed as the number of cells per square millimeter. During cell counting, no attempt was made to distinguish RGCs from displaced amacrine cells or other neuronlike cells. However, morphologically distinguishable gial cells, degenerated cells, and vascular endothelial cells were excluded from the cell counts. All quantification was performed in a masked manner. For each time point of each test condition, four animals were used.

**Apoptotic Cell Count**

Apoptotic neurons in the retinal flat mounts were stained with TUNEL (Apop-Taq In Situ Apoptosis Detection Kit; Roche, Burford, Switzerland) according to the manufacturer’s protocol. The pretreated flat-mounted retinas were incubated in the nuclelease-free proteinase K working solution for 10 minutes at 37°C and then in the TUNEL reaction mixture for 60 minutes at 37°C in a dark, humidified chamber. After the slides were rinsed with 0.01 M PBS and mounted with buffer glycerol, fluorescent images were taken with a fluorescence microscope (Eclipse TE2000-U, Nikon Corp., Japan) at 400× magnification. The number of dead neurons in the 12 images was counted; each image was counted three times. All 36 counts were averaged to give a mean value for each retina, expressed as the number of cells per square millimeter. During cell counting, no attempt was made to distinguish RGCs from displaced amacrine cells or other neuronlike cells. However, morphologically distinguishable gial cells, degenerated cells, and vascular endothelial cells were excluded from the cell counts. All quantification was performed in a masked manner. For each time point of each test condition, four animals were used.

**Quantitative Reverse Transcription–Polymerase Chain Reaction**

Retinal levels of mRNA of NPRB, Bcl-xL, BAX, μ-calpain, and retinal RNA, 1 μL oligo(dT) 18 (0.5 μg/μL), and complimentary DEPC H₂O was incubated at 70°C for 5 minutes, followed by adding 4 μL 5X reaction buffer, 1 μL RNase inhibitor (20 U/μL), 2 μL dNTP mix (10 mM), and 1 μL M-MuLv reverse transcriptase (200 μL/μL). The total reaction mixture was incubated sequentially at 25°C for 10 minutes, 42°C for 1 hour, and 72°C for 2 minutes. The target cDNAs were amplified by PCR (MyCycler; Bio-Rad, Hercules, CA). PCR was performed in 30 μL of total reaction volume containing 2 μL template cDNA, 3 μL 10X PCR buffer, 3 μL MgCl₂ (25 mM), 3 μL dNTP mix (2.5 mM), 0.9 μL of 10 pM specific sense and antisense primers for the different genes (Table 1), and 0.3 μL DNA polymerase (5 U/μL; Taq; Applied Biosystems, Inc. [ABI], Foster City, CA). The cycling protocol consisted of one cycle of 3 minutes at 94°C followed by 35 cycles of denaturation for 10 seconds at 94°C, annealing for 30 seconds at the proper temperature (Table 1), extension for 30 seconds at 72°C, and then extension for 10 minutes at 72°C. Fluorescence quantitative PCR was performed (MyCycler; Bio-Rad). The total reaction mixture was the same as just stated, plus 0.2 μL 2× SYBR green (Invitrogen). The cycling protocol consisted of one cycle of 2 minutes at 94°C followed by 45 cycles of 10 seconds at 95°C, 10 seconds at 60°C, 40 seconds at 72°C, and 20 seconds at 84°C (for fluorescence collection and detection). The GAPDH mRNA level was used to normalize the mRNA levels of specific targets for each sample. Primers for specific cDNAs, synthesized by Invitrogen (Shanghai, China), are listed in Table 1.

**Statistical Analysis**

Data are presented as the mean ± SEM. Differences among three or more groups were analyzed by one-way ANOVA followed by Bonferroni tests (SPSS 15.0 statistic analysis software for Window, SPSS Inc., Chicago, IL). Results with P < 0.05 were considered statistically significant.

**RESULTS**

**Effects of Natriuretic Peptides on Cultured Rat RGC Survival**

To determine whether natriuretic peptides are neuroprotective, we evaluated ANP and CNP in the cultured rat retinal cell model with three different insults: glutamate, TNFα, and withdrawal of trophic factor. We chose these cytotoxic insults because they have been implicated in glaucomatous retinopathy with different molecular mechanisms. Under the conditions used in these studies, there were 100 to 200 RGCs per control well. Treatment with glutamate (100 μM), an excitatory amino acid, caused significant morphologic changes in many RGCs, including the loss of neurites, an increased number of vacuoles in the cytoplasm, and compromised integrity of the plasma membrane. Incubation with glutamate also reduced the number of RGCs in culture. Only approximately 60% of cells survived after a 3-day treatment (Figs. 1A, 1B). The effect had been shown to be mediated mainly by the NMDA...
receptor, since MK801 was the most efficacious antagonist. Incubation of the cells with CNP dose-dependently protected against glutamate-induced toxicity (Fig. 1B). Maximum protection was achieved by 50 nM CNP, where 88% (mean ± SEM, n = 6) of RGCs survived the insult, significantly different (P < 0.001) from RGC survival with glutamate alone (62%, n = 9; Fig. 1B). In contrast, ANP (5–500 nM) treatment did not produce significant protection (Fig. 1A). ANP or CNP by itself did not affect RGC survival (data not shown).

In addition to glutamate, the survival of RGCs in culture was also sensitive to TNFα (10 ng/mL); approximately 60% of cells survived after a 3-day treatment (Figs. 1C, 1D). Neither ANP nor CNP (5–500 nM) significantly protected the cells against this insult (Figs. 1C, 1D).

Removal of BDNF, bFGF, CNTF, and forskolin from the culture medium (trophic factor withdrawal) for 3 days caused a significant loss of RGCs. CNP partially but significantly protected the RGCs against this insult in a concentration-dependent manner. At 50 and 500 nM CNP, 75% ± 4% (n = 6; P < 0.05) and 76% ± 7% (n = 9; P < 0.01) of RGCs survived the trophic factor withdrawal-induced cytotoxicity, respectively, compared with 56% ± 3% (n = 9) survival with the insult alone (Fig. 1F). Again, ANP was not protective (Fig. 1E).

**Effects of NMDA and CNP on Inner Plexiform Layer Thickness**

The in vitro neuroprotective effect of CNP but not ANP on RGCs led us to assess the potential in vivo protective effects of CNP against excitotoxicity-induced RGC death. Excitotoxic amino acid NMDA, when injected intravitreally, causes morphologic changes and RGC loss in the rat retina. We confirmed these findings and further demonstrated that co-injection of CNP mitigated the changes. For example, Figure 2 shows representative HE-stained rat retinal sections at different time points, up to 14 days after different treatments. Intravitreal injection of PBS did not produce any observable changes in the retina, whereas 20 nanomoles of NMDA produced a time-dependent, obvious thinning of the inner plexiform layer (IPL) and an apparent reduction in cell density in the GCL. Other than that, NMDA injection did not induce any visible changes in the inner nuclear layer (INL), outer nuclear layer (ONL), and outer plexiform layer (OPL). These NMDA-induced retinal changes appeared to be diminished when CNP (10 μg, 4.5 nanomoles) was co-injected. Intravitreal injection of CNP alone did not affect the retina (Fig. 2).
To quantify the morphologic observations, we measured thickness of the IPL, INL, OPL, and ONL on rat cross-sections 1.0 to 1.5 mm from the margin of the optic nerve head. In agreement with the findings shown in Figure 2, the thickness of the INL, OPL, or ONL was not significantly affected by the various treatments (data not shown). In contrast, intravitreal injection of 20 nanomoles NMDA decreased IPL thickness, which was significantly different from that in PBS-injected eyes at the same time points, starting at 18 hours after injection and lasting for at least 14 days (Fig. 3). At 14 days, in the PBS-injected eyes, IPL thickness was 22.0 ± 1.1 μm (n = 4), whereas in the NMDA-injected eyes, it was 9.5 ± 0.4 μm (n = 4; P < 0.001). Coadministration of CNP with NMDA partially reduced the detrimental effect of NMDA, which achieved statistical significance on days 7 and 14. IPL thickness at day 14 in animals injected with both NMDA and CNP was 17.1 ± 0.04 μm (n = 4; P < 0.001 versus NMDA; Fig. 3). Injection of CNP itself did not significantly affect IPL thickness (21.8 ± 0.8 μm at day 14; n = 4; P > 0.05 versus PBS; Fig. 3).

**Effects of NMDA and CNP on GCL Cell Count**

As implicated by morphologic observations, the NMDA (20 nanomoles) injection appeared to decrease cell density in GCL in the retinal cross sections, which was partially reversed by CNP (4.5 nanomoles; Fig. 2). To confirm these observations, we stained rat retinal flat mounts with cresyl violet, to label cells in the GCL, and counted stained neuronlike cells in randomly selected images, each representing a 4.7 × 10⁻³ mm² area, of the central, midperipheral, and peripheral regions of all four quadrants. During cell counting, we were not able to distinguish RGCs from displaced amacrine cells or other neuronlike cells. However, morphologically distinguishable glial cells, degenerated cells, and vascular endothelial cells were excluded. Figure 4 shows representative images obtained at different time points of the different treatments.

Quantification of GCL neuron counts demonstrated that PBS injection itself did not have significant effects on cell survival up to 14 days (Fig. 5). However, NMDA (20 nanomoles) treatment significantly decreased neuron counts at all time points (P < 0.001 versus PBS). At 6 hours, NMDA-treated retinas had 25%
fewer cresyl violet–labeled neurons than did the PBS-treated retinas. This cell loss increased to 48% at 14 days (Fig. 5). Concomitant treatment with CNP (4.5 nanomoles) reduced the NMDA effect, especially at later time points. At 14 days, the retinas with both CNP and NMDA treatment had only 24% fewer cell counts relative to that of PBS-treated retinas (P < 0.001 versus PBS, P < 0.01 versus NMDA; Fig. 5). CNP (4.5 nanomoles) injection alone slightly decreased cresyl violet–labeled neurons. Compared with the PBS-treated retinas, CNP-injected retinas had 7% fewer cells at 14 days (P < 0.001 versus PBS; Fig. 5).

**Effects of NMDA and CNP on Retinal Neuron Apoptosis**

NMDA-induced death of retinal neurons very likely involves apoptosis. To assess this phenomenon, we labeled rat retina flat mounts at different time points after the various treatments with TUNEL and counted TUNEL-positive cells in the central, midcentral, and peripheral regions of all four quadrants. Figure 6 shows representative images. It is obvious that intravitreal injection of 20 nanomoles NMDA was associated with a larger number of TUNEL-positive cells, especially within the first 24 hours after treatment. In contrast, the other groups had far fewer cells with TUNEL staining.

Quantification of apoptotic GCL neuron counts demonstrated that PBS injection itself did not have significant effects on cell apoptosis up to 14 days (Fig. 7). However, intravitreal injection of 20 nanomoles NMDA induced a dramatically higher number of TUNEL-positive cells at all time points (P < 0.05 versus PBS), especially within the first 24 hours after treatment (P < 0.001 versus PBS) (Fig. 7). In contrast, CNP-treated (4.5 nanomoles) retinas had far fewer TUNEL-positive cells, similar to PBS-treated retinas at all time points (P > 0.05 versus PBS; Fig. 7). Furthermore, concomitant treatment with CNP (4.5 nanomoles) reduced the NMDA (20 nanomoles) effect with significantly fewer TUNEL-positive cells relative to that of only NMDA-treated retinas (P < 0.01 or 0.001 versus NMDA) at all time points (Fig. 7).

**Effects of NMDA and CNP on Retinal mRNA Expression of NPRB, Bcl-xL, BAX, and µ-Calpain**

To further characterize the NMDA and CNP effects, we analyzed the retinal mRNA levels of NPRB and molecules known to be involved in apoptosis—Bcl-xL, BAX, and µ-calpain—by qRT-PCR at three time points: 6 hours, 24 hours, and 4 days. Each sample contained a pool of three retinas. The mRNA levels of these specific targets in each sample were normalized against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level, and their normalized levels in the PBS-treated retina were set at 100%.

There was no significant difference in NPRB mRNA expression in the treatment groups at any time point (P > 0.05, ANOVA; Fig. 8A). In contrast, the mRNA levels of the apoptosis-related molecules Bcl-xL, BAX, and µ-calpain were elevated in the NMDA-treated (20 nanomoles) retinas, albeit with different temporal profiles. For example, a significant increase in Bcl-xL mRNA (625% ± 150%; P < 0.001 versus PBS) was observed only at 4 days after NMDA injection (Fig. 8B). A trend of elevated BAX mRNA levels was noted at 6 hours (254% ± 35%; P > 0.05, ANOVA) after NMDA treatment that became
statistically significant at 24 hours (475% ± 48%; P < 0.001 versus PBS) and at 4 days (537% ± 226%; P < 0.05 versus PBS; Fig. 8C). All these NMDA-induced changes were significantly reduced by concomitant treatment with CNP (4.5 nanomoles; P < 0.01–0.001 versus NMDA), whereas CNP (4.5 nanomoles) injection by itself did not significantly affect the mRNA levels of these molecules (Figs. 8B–8D).

**DISCUSSION**

Excitotoxicity, deprivation of trophic factors, and TNFα have been proposed as possible mechanisms underlying RGC death in glaucoma.4–7,9 In this report, we found that glutamate, TNFα, and withdrawal of trophic factor jeopardized the survival of cultured adult rat RGCs, and similar to previous findings, the insults at their maximum effects caused the death of approximately half of the RGCs.35 At the present time, it is not clear whether the neurons surviving these insults differed biologically or functionally from those that perished. ANP, at 500 nM, showed a trend of protection (approximately 10%) against glutamate- and TNFα-induced cell death, but the effect was not statistically significant. In contrast, CNP conferred a partial yet significant protection against both glutamate- and trophic factor withdrawal–induced cytotoxicity. The maximum protection afforded appeared to be better against excitotoxicity. In fact, RGC survival in the presence of both CNP (50 nM) and glutamate was not significantly different from the no-insult control group (P > 0.05). The different efficacies of CNP protection in cell survival against the different insults confirm that different mechanisms of cytotoxicity are evoked by these insults, and CNP is effective in modulating some of the mechanisms, such as excitotoxicity, but not all of them.

Glutamate has been shown to play a role in the pathologic course of various neurodegenerative diseases, including retinal ischemia,38 diabetic retinopathy,39 optic nerve ischemia,40 and even stroke and epilepsy.41 In glaucoma, it has been suggested that the initial damage to retinal cells induces an excessive release of glutamate, which subsequently damages neighboring RGCs, induces more glutamate release, and perpetuates the feed-forward cycle.42 Because of the potential involvement of glutamate in glaucoma and the in vitro CNP results presented herein, we chose to investigate the potential protective effects of CNP against intravitreal NMDA-induced retinal toxicity in vivo.

In this study, we found that intravitreal injection of 20 nanomoles NMDA decreased the thickness of IPL and the number of neurons in the GCL of the rat. These results are consistent with those reported in other studies.43–46 These excitotoxic effects were all partially reversed by treatment with 4.5 nanomoles (10 μg) CNP. CNP protected against the NMDA-induced time-dependent thinning of IPL. At day 14 of NMDA injection, the thickness of IPL was reduced to 43% of that of the vehicle control, whereas when CNP was added, the IPL thickness was significantly improved to 77% of control. This finding correlated with a partial protection by CNP against...
the NMDA-induced reduction in GCL cell density. Again, at day 14 after NMDA treatment, the GCL cell density was reduced to 52% of the control value, and CNP significantly, although incompletely, mitigated the NMDA effect. Raising the GCL cell density to 76% of control CNP injection by itself did not affect the thickness of IPL. It did significantly reduce the GCL cell density (9%). The biological implication of this minor change is uncertain at this time.

In addition to morphologic changes, intravitreal injection of 20 nanomoles NMDA also induced apoptosis of RGCs. It has been reported that apoptosis of the RGCs often appears at 18 to 24 hours after NMDA injection and is sustained for 4 to 14 days.32,37,45–47–50 We demonstrated that apoptosis of GCL neurons induced by 20 nanomoles NMDA occurred as early as 6 hours and peaked at 24 hours after injection. However, minimal RGC apoptosis was noticed at day 4 and thereafter, even though the number of cells in the GCL decreased, at least until day 14. This result suggests that the neurons in this layer were dying continually, probably due to the secondary neuronal death. Our study showed that 4.5 nanomoles (10 μg) CNP protected the neurons in GCL from 20 nanomoles NMDA-induced degeneration throughout the study period, from 6 hours to 14 days. CNP itself, similar to the vehicle control, did not induce significant apoptosis of retinal neurons.

These morphologic and biochemical analyses clearly indicate the protective effects of CNP against excitotoxicity in the retina, but its molecular mechanism of action remains unclear. Excitotoxicity in the retina has been proposed to involve excessive Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) overload of the RGC, leading to μ-calpain activation and apoptotic cell death.45–46 Calpains, such as μ-calpain (calpain 1) and m-calpain (calpain 2), belong to a family of Ca\(^{2+}\)-dependent cysteine proteases and are ubiquitous in the central nervous system (CNS), including the RGCs.45 μ-Calpain, but not m-calpain, when activated by stresses such as Ca\(^{2+}\) overload, induces proteolysis in the cell, which has been implicated as a major process in pathologic neuronal cell death in many neurodegenerative disorders—excitotoxicity in the retina, for example.45,51–53 CNP activates NPRB and stimulates intracellular cGMP production. cGMP affects the functions of several downstream signaling molecules. For example, it activates cGMP-dependent protein kinase (also known as protein kinase G) and cyclic nucleotide-gated ion channels, which in turn increases K\(^{-}\) efflux, causes membrane hyperpolarization, reduces Ca\(^{2+}\) influx, and suppresses Ca\(^{2+}\) release from the endoplasmic reticulum. cGMP also decreases various signaling systems’ responsiveness to Ca\(^{2+}\).52,17 A cGMP-mediated suppression of Ca\(^{2+}\) influx, release, and responsiveness may therefore be responsible, at least in part, for the neuroprotective effects of CNP on these cells.

To attempt to elucidate the molecular effects of NMDA and CNP, we evaluated the retinal expression of μ-calpain, as well as that of Bcl-2 and BAX, genes products involved in the cell apoptosis cascade.54,55 after intravitreal injection of the compounds. Our findings showed that 20 nanomoles NMDA increased mRNA expression of μ-calpain in the retina at 6 hours after treatment, but the level returned to baseline at later time points. NMDA also increased the expression of both BAX and Bcl-XL. It induced an increase of BAX expression in a time-dependent manner. The increased expression of BAX mRNA was noted at 4 days after injection, suggesting that the pro-apoptotic BAX gene was still turned on by the insult when there was minimal RGC apoptosis at day 4 and thereafter. Of note, the mRNA level of Bcl-XL followed a different temporal pattern. It was not elevated until 4 days after NMDA treatment. These data implicate a temporal sequence of molecular events initiated by NMDA, from the increased expression of μ-calpain, leading to BAX and Bcl-XL activation. These effects of NMDA were completely blocked by the co-injection of 4.5 nanomoles (10 μg) CNP.

In the same study samples, we assessed the mRNA levels of NPRB. Consistent with prior findings that the inner retina expresses CNP and NPRB,21,26,27,56,57 we demonstrated the presence of NPRB in the rat retina by qRT-PCR, which was subsequently confirmed by Western blot analysis (Ma et al., unpublished observations, 2009). All treatments tested did not generate significant changes in the levels of mRNA or protein of NPRB, indicating that CNP or NMDA did not up- or down-regulate the receptor.

In summary, we confirmed previous findings that glutamate, TNFα, and withdrawal of trophic factors reduce survival...
of adult rat RGCs in culture. We also confirmed that intravitreal injection of NDMA induces time-dependent changes in the inner retina, such as loss of neurons in the GCL; an increase in TUNEL-positive nuclei; an increased expression of apoptosis-related genes including Bcl-xL, BAX, and μ-calpain, and thinning of the IPL. Most important, we demonstrated that CNP, but not ANP, dose-dependently protected against glutamate- and trophic factor withdrawal-induced cytotoxicity in cultured adult rat RGCs. We further showed that intravitreal injection of CNP ameliorated excitotoxic amino acid-induced damage in the retina. These in vitro and in vivo neuroprotective effects of CNP against excitotoxicity in RGC are intriguing. Moreover, CNP is known to lower IOP. It is likely that CNP holds a potential to be used as an antiglaucoma medication. Unfortunately, being a peptide, CNP is not efficacious after topical administration. Currently, the most efficient method of delivering CNP is by intravitreal injection, but this approach in glaucoma treatment is not clinically practical. However, the gene therapy that is under development in our and other laboratories may resolve this problem.

References


