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Research Report

Neuroprotective effect of atrial natriuretic peptide against NMDA-induced neurotoxicity in the rat retina

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ARTICLE INFO

Article history:
Accepted 22 November 2005
Available online 26 January 2006

Keywords:
Atrial natriuretic peptide
N-methyl-D-asparate
Dopamine
Tyrosine hydroxylase
Retina
Neuroprotection

ABSTRACT

Atrial natriuretic peptide (ANP) can regulate aqueous humor production in the eye and has recently been suggested to play some functional roles in the retina. It has also been reported that ANP increases tyrosine hydroxylase (TH) mRNA levels and intracellular dopamine levels in PC12 cells. The effect of ANP on TH levels and the role of ANP in retinal excitotoxicity remain unknown. In this study, we investigated the effects of ANP on TH expression and dopamine levels in rat retina after intravitreal injection of NMDA. Immunohistochemistry localized natriuretic peptide receptor-A (NPRA) in the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL) in the rat retina. Quantitative real-time PCR and Western blot analysis showed a dramatic reduction in retinal TH levels 5 days after NMDA injection, while ANP, at a concentration of 10^{-4} M, ameliorated this reduction in TH mRNA and TH protein levels. High-performance liquid chromatography (HPLC) analysis showed that NMDA reduced dopamine levels in the retina, and that ANP attenuated this reduction. Moreover, morphological analysis showed that ANP ameliorated NMDA-induced neurotoxicity through NPRA. The ameliorative effect of ANP was inhibited by a dopamine D_1 receptor antagonist. These results suggest that ANP may have a neuroprotective effect through possible involvement of dopamine induction.

1. Introduction

Excitotoxicity, such as glutamate-induced neuronal cell death, has been linked to some ocular diseases, including retinal ischemia (Louzada-Junior et al., 1992; Neal et al., 1994), diabetic retinopathy (Kowluru et al., 2001), and optic neuropathy (Kim et al., 2000; Yoles and Schwartz, 1998). Excessive activation of *N*-methyl-D-aspartate (NMDA) receptors, a glutamate receptor subtype, causes degeneration of neuronal cells in the retina (Dkhissi et al., 1999; Facci et al., 1990; Gibson and Reif-Lehrer, 1985). Intravitreal injection of NMDA results in not only loss of retinal ganglion cells (RGCs) (Siliprandi et al., 1992) but also loss of retinal dopaminergic neurons (Kitaoka et al., 2003).

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doi:10.1016/j.brainres.2005.11.068

Dopaminergic amacrine cells contain tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and the following two types of TH immunoreactive cells have been identified: type I, which is large and located in the inner nuclear layer (INL), and type II, which is small and located more deeply within the INL (Wu and Cepko, 1993). Dopamine can inhibit NMDA receptor activity in neurons from the chick retina (Castro et al., 1999) and protect cultured rat retinal neurons against NMDA receptor-mediated glutamate neurotoxicity through the dopamine D_1 receptor (Kashii et al., 1994). Therefore, alteration of TH or dopamine levels may influence NMDA-induced retinal neurotoxicity.

Natriuretic peptides (NPs) consist of three isoforms, termed atrial NP (ANP) (de Bold, 1985), brain NP (BNP) (Sudoh et al., 1988), and C-type NP (CNP) (Sudoh et al., 1990). The biological actions of these NPs are mediated by membrane-bound receptors containing a guanylyl cyclase (GC) domain with intracellular cGMP as a second messenger. NP receptors have been classified as NP receptor-A (NPRA) (Chinkers et al., 1989; Lowe et al., 1989; Nathanson, 1987), NPRB (Chang et al., 1989; Schulz et al., 1989), and NPRC (Fuller et al., 1988; Schenk et al., 1989; Shimonaka et al., 1987). Two of these receptor subtypes, NPRA and NPRB, are members of the family of membrane-bound GC and have four domains: an N-terminal extracellular ligand-binding domain, a transmembrane domain, a kinase-like regulatory domain, and a C-terminal GC domain (Chinkers and Garbers, 1991). Most of the physiological actions of NPs are mediated by these receptor GCs through the generation of the second messenger, cGMP. NPRA has a higher affinity for ANP than BNP, whereas ANP and BNP have little effect on NPRB, which is activated preferentially by CNP (Schulz et al., 1989). In the eye, Fernandez-Durango et al. showed that NPRAs were localized in the retina, choroid and ciliary process of the rat and rabbit eye (Fernandez-Durango et al., 1989, 1995). Although several reports have shown that ANP can regulate aqueous humor production in the eye (Bianchi et al., 1986; Mittag et al., 1987; Nathanson, 1987, Tjalve and Wilander, 1988), its effect on the retina remains to be elucidated. On the other hand, it has been reported that ANP increases TH mRNA levels and intracellular dopamine levels in PC12 cells (Takekoshi et al., 2000). Thus, in the present study, we investigated the effects of ANP on TH expression and dopamine levels in the rat retina after intravitreal injection of NMDA.

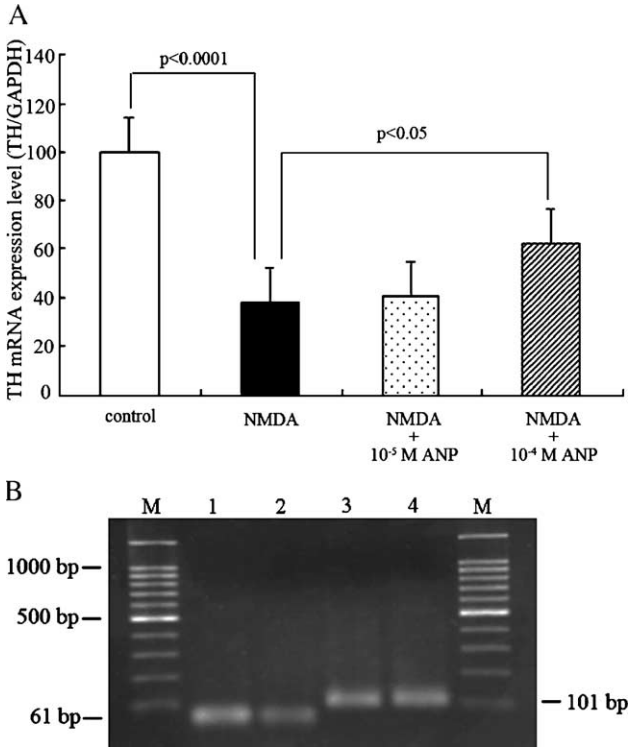


Fig. 2 – (A) Real-time polymerase chain reaction analysis of tyrosine hydroxylase (TH) mRNA levels in the rat retina 5 days after intravitreal injection of phosphate-buffered saline (PBS), 200 nmol *N*-methyl-D-aspartate (NMDA), or 200 nmol NMDA + 0.05 nmol atrial natriuretic peptide (ANP) or 0.5 nmol ANP. Data are normalized to glyceral-dehyde-phosphate dehydrogenase (GAPDH) levels in the same sample and expressed as a percentage of the control value. Each column represents the mean ± SEM (n = 8–9). (B) Electrophoretic analysis of real-time PCR products amplified from the rat retina with primers specific for TH (lanes 1 and 2, 61-bp product) and GAPDH (lanes 3 and 4, 101-bp product). M indicates 100-bp ladder standards.

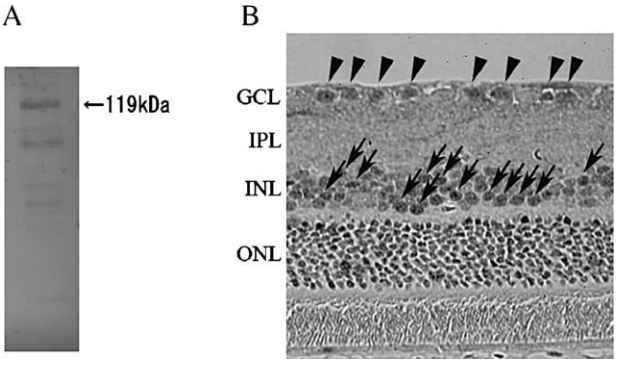


Fig. 1 – (A) Western blot analysis showed the existence of the ANP-receptor (NPRA) in the intact rat retina. The NPRA band had a molecular weight of 119 kDa. (B) Immunohistochemistry of NPRA in the rat retina. The NPRA immunostaining was observed in the ganglion cell layer (GCL) (arrowheads), the inner nuclear layer (INL) (arrows), and the outer nuclear layer (ONL) (abundant NPRA immunostaining).

2. Results

2.1. *Confirmation of existence of NPRA in the rat retina*

Western blot analysis showed that there was NPRA in the rat retina (Fig. 1A). We verified the presence of NPRA band at 119 kDa in samples from the rat retina. The immunohistochemical study showed an abundance of NPRA immunopositive cells in the ganglion cell layer (GCL), the INL, and the outer nuclear layer (ONL) in the rat retina (Fig. 1B).

2.2. *Effect of ANP on NMDA-induced changes in TH levels in the retina*

Since we previously observed that NMDA induced time-dependent decreases in TH mRNA and TH protein levels (at days 3 and 5 after NMDA injection), we conducted this study 5 days after injection. In this study, quantitative real-time PCR of TH mRNA after reverse transcription showed a dramatic reduction in retinal TH levels 5 days after NMDA injection while ANP, at concentration of 10^{-4} M, reduced this decrease in TH mRNA (Fig. 2A). Products from completed quantitative real-time PCR runs were used to confirm specific amplification of TH or GAPDH cDNA, showing corresponding specific single bands by agarose gel electrophoresis with ethidium bromide staining and UV transillumination (Fig. 2B).

Western blot analysis showed that NMDA induced decreases in TH protein levels in the retina. Densitometry of those blots showed a significant decrease in the expression of the TH protein in the NMDA-treated retina at 5 days, as shown previously. In this study, we observed that the reduction in TH protein levels by NMDA was significantly attenuated by ANP (Figs. 3A, B). However, ANP did not significantly alter the TH protein levels in intact rats (Fig. 3C).

2.3. *Effect of ANP on NMDA-induced changes in dopamine levels in the retina*

Dopamine levels in the rat retina were examined 5 days after intravitreal injection of PBS, NMDA, or NMDA + 10^{-5} or 10^{-4} M ANP by HPLC. The basal dopamine level in the retina of control eyes was 4.26 ± 0.94 ng/mg tissue. NMDA significantly reduced dopamine levels to approximately 18.5% of the control level. This decrease in the level of dopamine was significantly attenuated by ANP (Fig. 4).

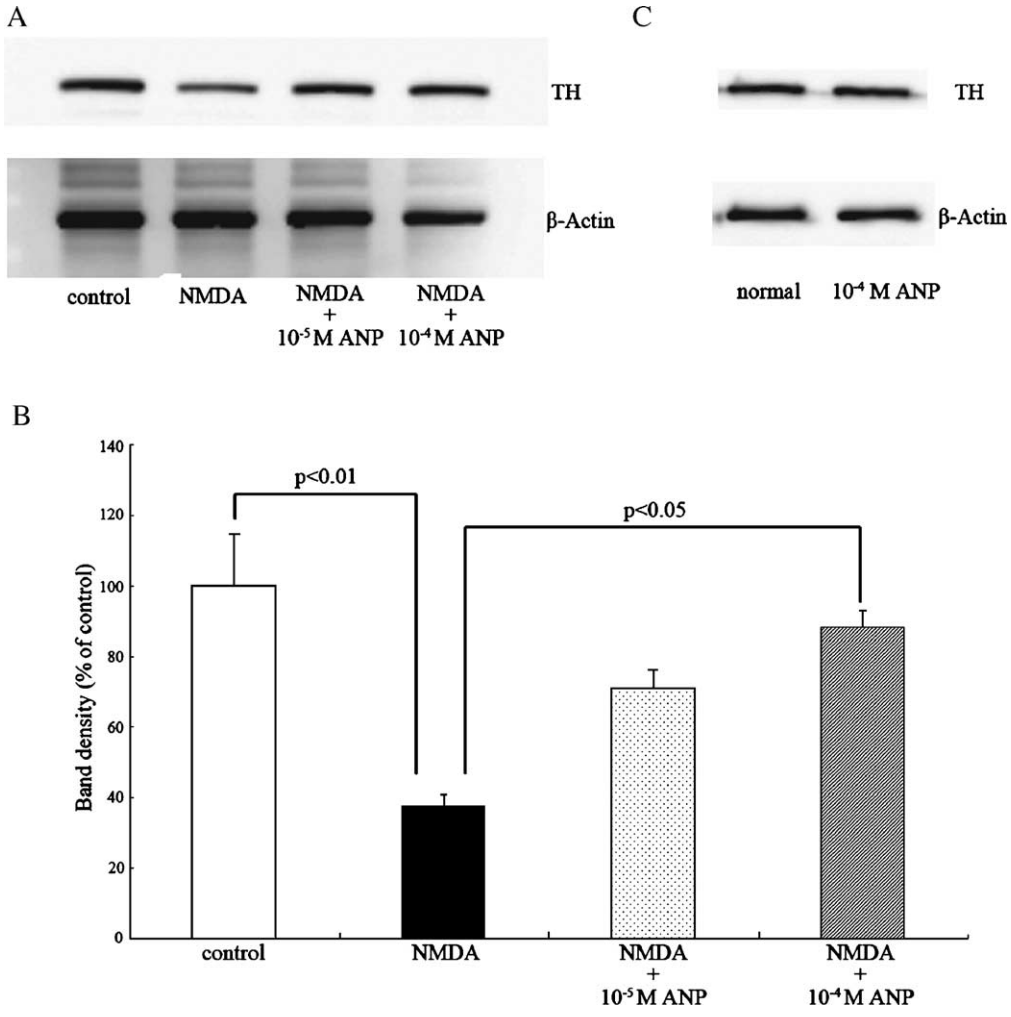


Fig. 3 – (A) Western blot analysis of tyrosine hydroxylase (TH) protein levels in the rat retina 5 days after intravitreal injection of phosphate-buffered saline (PBS), 200 nmol *N*-methyl-D-aspartate (NMDA), or 200 nmol NMDA + 0.05 nmol atrial natriuretic peptide (ANP) or 0.5 nmol ANP. (B) Densities of the immunoreactive bands were analyzed by a densitograph gel documentation system (ATTO-densitograph, Osaka, Japan). Data are expressed as a percentage of the control value. Each column represents the mean ± SEM (n = 7). (C) Western blot analysis of TH protein levels in the rat retina from intact eyes (normal eyes) or 5 days after intravitreal injection of 0.5 nmol ANP (n = 5).

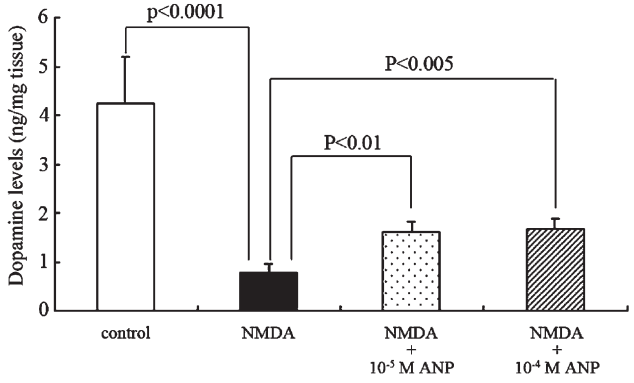


Fig. 4 – Dopamine levels in the rat retina 5 days after intravitreal injection of phosphate-buffered saline (PBS), 200 nmol *N*-methyl-D-aspartate (NMDA), 200 nmol NMDA + 0.05 nmol atrial natriuretic peptide (ANP), or 0.5 nmol ANP. Each column represents the mean ± SEM (n = 6–8).

2.4. *Effect of ANP on NMDA-induced histological changes in the retina*

Histological examination of retinal sections showed an ameliorative effect of ANP (Fig. 5A). Compared to the control, NMDA induced a loss of inner retinal elements showing a loss of cells in the GCL and thinning of the inner plexiform layer (IPL) as described previously. Interestingly, retinas to which ANP (10^{-5} or 10^{-4} M) was administered simultaneously with NMDA showed better preserved inner retinas with thicker inner retinas relative to those treated only with NMDA. After NMDA injection, both the number of cells in the GCL and the IPL thickness showed significant losses, but significantly higher cell numbers (Fig. 5B) and a thicker IPL (Fig. 5C) were observed in retinas treated simultaneously with NMDA and ANP. Moreover, the effects of ANP on NMDA-induced changes were inhibited by pre-injection of an anti-NPRA antibody (Figs. 5B, C). To examine the involvement of dopamine on the effect of ANP, we used two selective dopamine receptor antagonists. The protective effect of ANP on NMDA-induced damages was attenuated by pre-injection of *R*-(+)-SCH 23390, a dopamine D_1 receptor antagonist, while domperidone, a dopamine D_2 receptor antagonist, did not significantly alter the effect of ANP (Figs. 5B, C).

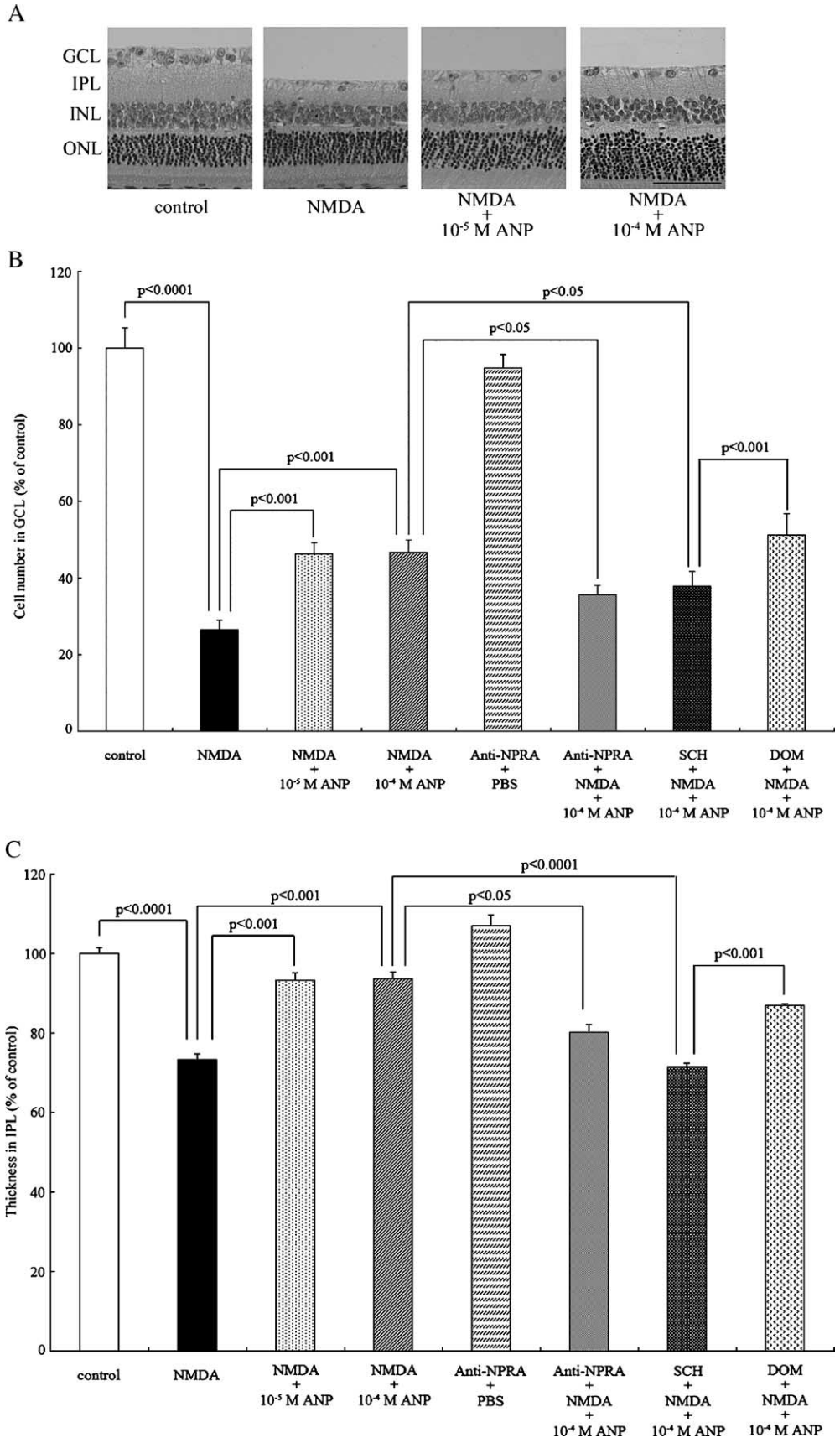
3. Discussion

In this study, we demonstrate that there is substantial NPRA protein in the rat retina. Moreover, we show that ANP inhibits reductions in TH and dopamine levels after intravitreal injection of NMDA. Furthermore, ANP ameliorates inner retinal neuronal cell loss as measured by morphometry. On the other hand, administration of an anti-NPRA antibody or *R*-(+)-SCH 23390 inhibits the effects of ANP on inner retinal neuronal cell loss. These results are consistent with our hypothesis that ANP may have a neuroprotective effect with the possible involvement of dopamine.

ANP has been shown to regulate aqueous humor production in the eye (Bianchi et al., 1986; Mittag et al., 1987; Nathanson, 1987; Tjalve and Wilander, 1988) and decrease intraocular pressure (Fernandez-Durango et al., 1999). Immunostaining of ANP has been demonstrated in the outer plexiform layer and IPL of rats and rabbits (Palm et al., 1989). Moreover, expression of NPRA (Ahmad and Barnstable, 1993; Fernandez-Durango et al., 1995; Kutty et al., 1992), NPRB, and NPRC mRNA has been reported in the rat retina (Fernandez-Durango et al., 1995). Consistent with these reports, we demonstrate the presence of NPRA protein in the rat retina by Western blot analysis. We also show that NPRA protein is localized in the GCL, INL, and ONL in retina by immunohistochemistry. Thus, it is likely that ANP mainly exists in synapse processes, and that its receptors are located in cell bodies. Recent studies have shown that retinal ANP concentrations in diabetic rats are significantly diminished relative to levels in control rats (Rollin et al., 2005). These results suggest that ANP may not only modulate aqueous humor production but may also have a putative functional role in retinal neurons.

In this study, we demonstrate that ANP significantly ameliorates NMDA-induced decreases in cell numbers in the GCL and thickness in the IPL of the rat retina. In addition, our morphometric study shows that inhibition of NPRA prevents the protective effects of exogenous ANP. Therefore, it is possible that ANP may exert its protective effect through NPRA. Interestingly, ANP inhibits apoptotic DNA fragmentation and prolongs the survival of serum-deprived PC12 cells with cGMP elevations (Fiscus et al., 2001). Moreover, ANP may contribute to cortical spreading depression-induced neuroprotection against ischemic insult via effects on cGMP production (Wiggins et al., 2003). These findings suggest that ANP may have neuroprotective potential in certain conditions. However, downstream effectors of ANP that are associated with this process remain unclear. NPs can increase intracellular levels of cGMP through two distinct signal transduction pathways in the retina (Blute et al., 2000). Increases in cGMP levels have been linked to protective actions in different neuronal cell types (Barger et al., 1995; Fiscus et al., 2001; Forloni et al., 1997; Wiggins et al., 2003). These may provide an opportunity for further studies to advance the understanding of the effects of ANP.

Previous studies have demonstrated that ANP induces increases in TH mRNA levels and dopamine levels in PC12 cells (Takekoshi et al., 2000). In this study, the decreases in TH mRNA, TH protein, and dopamine levels after NMDA injection were ameliorated by co-injection of ANP. The precise mechanisms by which ANP increases TH mRNA remain unclear, although involvement of cGMP and some transcription factors has been suggested (Takekoshi et al., 2000). Since TH is the rate-limiting enzyme in dopamine synthesis, increases in TH levels result in increases in dopamine levels. Dopamine is a chemical mediator that can behave as either a neurotransmitter or a neuromodulator in different regions of the central nervous system. Some of the effects of endogenous dopamine involve modulation of glutamate receptor function (Castro et al., 1999). Because dopamine has been reported to protect retinal neurons against NMDA receptor-mediated glutamate neurotoxicity (Kashii et al., 1994; Yamauchi et al., 2003), increases in TH expression and subsequent dopamine induction may lead to neuroprotection (Kitaoka and Kumai, 2004; Kitaoka et al., 2003). Our morphometric findings that the


A
GCL
IPL
INL
ONL
control
NMDA
NMDA + 10-5 M ANP
NMDA + 10-4 M ANP
B
Cell number in GCL (% of control)
p<0.0001
p<0.05
p<0.05
p<0.001
p<0.001
p<0.001
control
NMDA
NMDA + 10-5 M ANP
NMDA + 10-4 M ANP
Anti-NPRA + PBS
Anti-NPRA + NMDA + 10-4 M ANP
SCH + NMDA + 10-4 M ANP
DOM + NMDA + 10-4 M ANP
C
Thickness in IPL (% of control)
p<0.0001
p<0.001
p<0.0001
p<0.001
p<0.05
p<0.001
control
NMDA
NMDA + 10-5 M ANP
NMDA + 10-4 M ANP
Anti-NPRA + PBS
Anti-NPRA + NMDA + 10-4 M ANP
SCH + NMDA + 10-4 M ANP
DOM + NMDA + 10-4 M ANP

protective effect of ANP was inhibited by R-(+)-SCH 23390 imply that its effect may be exerted through the dopamine D_1 receptor. It is interesting to note that nicotine, which can increase TH expression (Hiremagalur et al., 1993), also has a protective action in retinal neurons against glutamate neurotoxicity through dopamine release with dopamine D_1 receptor involvement (Yasuyoshi et al., 2002). Taken together, our findings suggest that ANP-induced increases in TH expression and dopamine levels may be involved in its protective actions.

In summary, ANP ameliorated decreases in TH mRNA, TH protein, and dopamine levels in NMDA-induced retinal neurotoxicity. ANP may possess a neuroprotective effect against NMDA-induced excitotoxicity with possible involvement of dopamine D_1 receptor.

4. Experimental procedure

4.1. Animals

Experiments were performed on 8-week-old male Wistar rats. All studies were conducted according to the Association for Research in Vision Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed in a room where temperature (23 ± 1 °C), humidity (55 ± 5%), and lighting (light from 06:00 to 18:00 h) were controlled.

4.2. Immunohistochemistry

The immunohistochemical study was performed with samples from three rats. Intact rats were sacrificed by intraperitoneal (i.p.) injection of sodium pentobarbital. The eyes were enucleated, fixed by immersion in 10% neutral-buffered formalin for 24 h, dehydrated, and embedded in paraffin. Immunochemistry was performed using the diaminobenzidine (DAB) detection method on paraffinized retinal sections (4 μm thick). The primary and secondary antibodies were an anti-NPRA antibody (Abgent San Diego, CA) diluted 1:200 in PBS and an anti-rabbit antibody (DAKO EnVison Systems, DakoCytomation, CA), respectively. Negative controls were performed by replacing the primary antibody with PBS or serum. Each slide was processed with DAB reaction for 3 min.

4.3. Animal models of NMDA-induced neurotoxicity

Intravitreal injection of NMDA (Sigma, St. Louis, MO) was performed as described previously (Isenoumi et al., 2004; Kitaoka et al., 2003; Mizuno et al., 2001; Siliprandi et al., 1992). Briefly, rats were anesthetized by i.p. injection of sodium pentobarbital (35 mg/kg). Body temperature was maintained at 37 °C with a heating pad (PS-su 100; Riken Kaihatsu, Tokyo, Japan) during the experiments. A single 5-μl injection of 4×10^{-2} M NMDA in 0.1 M PBS (pH 7.40) with or without 10^{-5} M or 10^{-4} M ANP (PEPTIDE institute, Inc. Osaka Japan), or PBS (control) was administered intravitreally into one eye. 10^{-4} M R-(+)-SCH 23390 (D_1 receptor antagonist; Sigma) or 10^{-4} M domperidone (D_2 receptor antagonist; Sigma) was administered 3 h before the above injections. R-(+)-SCH 23390 and domperidone were dissolved in DMSO to obtain a 10^{-2} M solution, and they were each diluted with 0.1 M PBS. Besides that, we used an anti-NPRA antibody as a specific competitor. A single 5-μl injection of 0.25 μg/μl anti-NPRA antibody was administered intravitreally 3 h before intravitreal injections of PBS or NMDA + 10^{-4} M ANP. Rats were sacrificed 5 days after intravitreal injection by i.p. injection of sodium pentobarbital, and the eyes were enucleated.

4.4. Real-time PCR

The level of TH mRNA from the retina 5 days after injection of PBS, NMDA, or NMDA + ANP was determined with a real-time PCR system (ABI Prism 7000, Applied Biosystems, Foster City, CA) as described previously, with a slight modification (Kitaoka et al., 2003). Forty rats were used for real-time PCR, and each sample contained one retina. Five days after injection, retinas were removed, and total RNA was isolated by acid guanidinium phenol–chloroform extraction using a single-step method (Chomczynski and Sacchi, 1987). Two micrograms of total RNA was reverse-transcribed with 100 U Moloney murine leukemia virus reverse transcriptase (Ambion, Austin, TX) in a 20 μl reaction volume containing RT buffer, random decamers, dNTP, and RNase inhibitor (Ambion). PCR was performed in a 25 μl total reaction volume containing 0.75 μl of cDNA, primers specific for TH or glyceraldehyde-phosphate dehydrogenase (GAPDH), and SYBR Green PCR Master Mix (Applied Biosystems), as described above. The primers for TH were 5′-TGTTGGCTGACCGCACAT-3′ (sense) and 5′-GGCCCCCAGAGATGCAA-3′ (antisense). The primers for GAPDH were 5′-TGAGGTGACCGCATCTTCTTG-3′ (sense) and 5′-TGGTAACCAGGCGTCCGATA-3′ (antisense). Serial dilutions of the standard templates were also used for parallel amplification. The threshold cycles (Ct) were calculated with ABI Prism 7000 SDS software (Applied Biosystems). Standard curves were plotted with Ct versus log template quantities. The quantities of samples were determined from standard curves. TH levels were normalized to those of GAPDH in each sample.

4.5. Western blot analysis

Western blot analysis was performed with samples from 37 rats, as described previously (Isenoumi et al., 2004; Kitaoka et al., 2003). Briefly, 5 days after intravitreal injection, retinas were collected, homogenized, and then centrifuged at 15,000 × *g* for 15 min at 4 °C. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, and CA). Protein samples (35 μg each) were subjected to SDS-PAGE in 10% polyacrylamide gels. The samples were transferred to enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) membranes, and the membranes were blocked with Tris-buffered saline (TBS)–0.1% Tween-20 containing 5% skim milk. The membranes were then incubated for 2 h with an anti-TH antibody (Chemicon, Temecula, CA) diluted 1:200 in TBS, an anti-NPRA antibody (Abgent, San Diego, CA) diluted 1:100 in TBS, or an anti-β-actin antibody (Sigma, St. Louis, MO) diluted 1:1000 in TBS. After three washes with 0.1% Tween-20 in TBS (T-TBS), the membranes were incubated for 1 h with peroxidase-labeled an anti-rabbit IgG antibody (Cappel, Aurora, OH) diluted 1:5000 or an anti-mouse IgG antibody (Cappel, Aurora, OH) diluted 1:1000 in T-TBS. After three washes with T-

Fig. 5 – (A) Representative light microscopic photographs of the rat retina 5 days after intravitreal injection of phosphate-buffered saline (PBS), 200 nmol N-methyl-D-aspartate (NMDA), or 200 nmol NMDA + 0.05 nmol atrial natriuretic peptide (ANP) or 0.5 nmol ANP. Scale bar = 50 μm. (B) The effects of ANP on NMDA-induced retinal neurotoxicity, and of the anti-NPRA antibody, 0.5 nmol R-(+)-SCH 23390 (SCH), or 0.5 nmol domperidone (DOM) on ANP in terms of cell number in the ganglion cell layer. Each column represents the mean ± SEM (n = 6–8). (C) Effects of ANP on NMDA-induced retinal neurotoxicity, and of the anti-NPRA antibody, SCH, or DOM on ANP in terms of the thickness of the inner plexiform layer. Each column represents the mean ± SEM (n = 6–8).

TBS, the immune complex was visualized with an ECL detection system (ECL Plus Western Blotting Detection Reagents, Amersham Pharmacia Biotech).

4.6. *Morphometric analysis*

Morphometric analysis was performed with samples from 28 rats, as described previously (Isenoumi et al., 2004; Kitaoka et al., 2004; Munemasa et al., 2005). Five days after intravitreal injection, the eyes were enucleated and fixed by immersion in Carnoy's fixative for 3 h at 4 °C, followed by dehydration and paraffin embedding. Transverse sections (4 μm thick) were made through the optic disc. The sections were stained with hematoxylin and eosin, and the images of each section were acquired with a light microscope (Eclipse TE300; Nikon, Tokyo, Japan) and a digital camera (Nikon). The number of cells in the GCL and the thickness of the IPL for a length of 500 μm were determined at 1.0 mm from the edge of the optic disc. Data from three sections from each eye were averaged for one eye, and at least eight eyes per experimental condition were used for analysis. All quantification was performed in a blinded manner.

4.7. *Dopamine levels*

Samples from 16 rats were used for the dopamine assay. Each retina was homogenized in 150 μl ice-cold buffer containing 0.05 M perchloric acid and 5 ng of dihydroxybenzylamine as an internal standard. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C, and the supernatant was mixed with 10 mg of aluminum oxide and 500 μl of 2 M Tris–EDTA, pH 8.7, for 15 min. The sedimented aluminum oxide was washed with 1 ml of 16.5 mM Tris–EDTA (pH 8.1), dried, and mixed with 300 μl of solvent (100% acetic acid–10% sodium metabisulfite–5% EDTA–water = 0.1:0.05:0.05:9.8) for 15 min, followed by centrifugation at 1800 × *g* for 1 min. The supernatant was passed through a 0.22-μm filter, and an aliquot (10 μl) was injected into a high-performance liquid chromatograph (HPLC; 510 Pump; Waters, Milford, MA) with an electrochemical detector (460 Detector; Waters), and a column (Cosmosil 5C18-AR Packed Column, 4.6 × 150 mm; Nakalai Tesque, Kyoto Japan). The mobile phase consisted of 50 mM sodium acetate, 20 mM citric acid, 3.75 mM sodium octyl sulphate, 1 mM di-*n*-butylamine, 0.134 mM EDTA, and 5% (v/v) methanol. All separations were performed isocratically at a flow rate of 0.9 ml/min at 35 °C. The detector potential was maintained at +0.65 V.

4.8. *Statistics*

Data represent the mean ± SEM. Differences among groups were analyzed by one-way analysis of variance (ANOVA), followed by Scheffe's method or the Mann–Whitney's method. A probability value of less than 0.05 was considered statistically significant.

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