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Neuroprotective effects of C3 exoenzyme in excitotoxic retinopathy

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Abstract

The purpose of this study is to evaluate the neuroprotective effects of C3 exoenzyme (C3) on N-methyl-D-aspartate (NMDA)-induced retinopathy in rats. C3 was expressed in *Escherichia coli* and purified by affinity chromatography. Immunofluorescence was performed in NIH 3T3 cells treated with C3 to verify the cellular uptake of the protein. NMDA was injected intravitreally into rat eyes with or without C3. At various time points after injection, eyes were enucleated. Hematoxylin/eosin staining was performed on retina cross-sections for morphological analysis. Survival and apoptosis of cells in the ganglion cell layer (GCL) were assessed by cresyl violet staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) on retina flat-mounts. RhoA levels in retina cells were evaluated by Western blot to detect C3 uptake in vivo. The cellular uptake of C3 was verified by immunofluorescence. Damage including a decrease in inner plexiform layer (IPL) thickness and reduction of cell density in the GCL, corresponding to apoptosis of neurons, was induced by intravitreal injection of NMDA. Protection against this damage was observed following co-injection of C3 and NMDA. RhoA ADP-ribosylation induced by C3 was confirmed by Western blot. Our results suggest that C3 exerts neuroprotective effects against excitotoxic damage induced by NMDA.

Keywords

glaucoma; neuroprotection; C3; excitotoxicity

1. Introduction

Glaucoma is an optic neuropathy and retinopathy with progressive retinal ganglion cell (RGC) death. To date, elevated intra-ocular pressure (IOP) is considered as the major risk factor for glaucoma (Toris, 2010). However, IOP lowering therapy often does not completely prevent RGC loss. Glaucomatous damage to RGCs can occur and progress despite significant IOP reduction (Baltmr et al., 2010). Like other neurons in the central nervous system, RGCs have limited capacity to survive or regenerate when damage occurs. Therefore, a neuroprotective approach aimed at delaying or preventing RGC death is a critical and active research area for the treatment of glaucoma.

At present, there is no neuroprotective agent approved for the treatment of this disease. Recently, a clinical trial conducted to test the efficacy of memantine (1-amino-3,5-dimethyladamantane), a non-competitive NMDA receptor antagonist prescribed for the treatment of Alzheimer's disease (Molinaro et al., 2009), as a neuroprotectant for glaucoma failed its primary endpoint (Danesh-Meyer and Levin, 2009). The explanation for the failure was not reported. However, an agent with a single mechanism of neuroprotection, such as memantine, may have limited efficacy in preventing RGC death in glaucoma (Osborne, 2009), in part due to the multifactorial pathogenesis of glaucoma. Consequently, pharmacological agents that simultaneously affect multiple mechanisms may have a better chance of success (Fang et al., 2010; Stroke Therapy Academic Industry, 1999). A randomized trial of brimonidine versus timolol in low-pressure glaucoma patients showed that topical treatment with brimonidine preserves visual fields better than treatment with timolol (Krupin et al., 2011). The underlying mechanisms remain unclear. Many molecules have been shown to be neuroprotective in various animal models such as optic nerve (ON) transection, crush, or ocular hypertension (Tamm et al., 2013).

C3 exoenzyme (C3) is a Rho inhibitor isolated from *Clostridium*. It was previously demonstrated to disrupt the actin cytoskeleton and cellular adhesions in human trabecular meshwork (TM) cells, and to increase outflow facility in organ cultured monkey anterior segments (Liu et al., 2005). This raised the possibility that C3 could be used as an IOP-lowering agent in glaucoma treatment. C3 also has other intriguing biological functions. For example, it was reported that treatment with C3 promoted axon regeneration (Fischer et al., 2004; Hu et al., 2007; Lehmann et al., 1999) and spinal cord repair (Dergham et al., 2002). This suggested the possibility that C3 might be used as a neuroprotective agent in glaucoma treatment.

In the current study, the neuroprotective effect of C3 on N-methyl-D-aspartate (NMDA)-induced excitotoxicity was evaluated in the rat retina.

2. Methods

2.1. C3 expression and purification

The C3 (GenBank accession no. X51464) cDNA was cloned into pET-26b (+) vector to generate a His-tagged fusion gene. BL21 (DE3) pLys^S competent cells, a strain capable of expressing non-toxic heterologous genes, were transformed with the new construct. A single

colony was inoculated into 100 ml of LB (Luria–Bertani) culture media containing 50 µg/ml kanamycin and incubated overnight at 37 °C with vigorous shaking. This culture was then added to 1 L of LB containing kanamycin and incubated at 37 °C until OD₆₀₀ = 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a concentration of 1 mM to induce protein expression. The culture was further incubated at 37 °C with shaking for an additional 5 h. After centrifugation at 4000 rpm for 10 min, the pellet was collected in 20 ml of ice-cold phosphate-buffered saline (PBS) containing 1% Triton X-100, sonicated for 30 min on ice, and centrifuged at 10,000× g for 20 min at 4 °C. The lysate was purified with a His-Bind resin cartridge (Bio-Scale Mini Profinity IMAC Cartridge, BIO-RAD, Hercules, CA, USA). Briefly, the cartridge was equilibrated with equilibration buffer (300 mM NaCl, 50 mM NaH₂PO₄, 5 mM imidazole, pH 8.0) before the sample was loaded. The cartridge was then rinsed with binding buffer (300 mM NaCl, 50 mM NaH₂PO₄, 5 mM imidazole, pH 8.0) and wash buffer (300 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, pH 8.0). C3 was eluted with elution buffer (300 mM NaCl, 50 mM NaH₂PO₄, 250 mM imidazole, pH 8.0). The eluted C3 was subjected to concentrating and desalting on an Amicon Ultra-15 filter column (Millipore, Billerica, MA, USA). The C3 solution was sterilized with a 0.22 µm filter. C3 concentration was determined using Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA) and the purification was verified as a single band on SDS-PAGE electrophoresis.

2.2. Cell culture and phalloidin staining

NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Thermo Scientific HyClone, Beijing), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mediatech), 100 µg/ml streptomycin, and 100 U/ml penicillin at 37 °C in 5% CO₂. Cells (1 × 10⁴ per 12 mm dish) were plated in 10% FBS/DMEM and treated with C3 at 30 µg/ml for 12 h.

Cells were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 h at room temperature. Phalloidin conjugated with FITC (Sigma–Aldrich, USA) was used to label the actin cytoskeleton, according to procedures described by the manufacturer. Images were captured using a fluorescence microscope (IX-71, Olympus Corp., Japan).

2.3. Immunofluorescence

Cells treated with C3 at 30 µg/ml for 12 h were washed once with PBS for 10 min and fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min at room temperature. Cells were then permeabilized in 0.25% Triton X-100 in PBS for 5 min and blocked with 10% NGS (normal goat serum) in PBS for 90 min. Primary mouse monoclonal antibody against His tag (TA-02, ZSGB-BIO, Beijing, China) was diluted at 1:300 with 3% NGS in PBS. Secondary antibody was applied with FITC-conjugated goat anti-mouse IgG (ZSGB-BIO, Beijing, China) at 1:200 dilution. Fluorescent images were acquired with a confocal fluorescence microscope (DSU, IX-71; Olympus, Japan) at 600× magnification.

2.4. Animals

All studies were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision

Research. Experiments were performed on adult male Sprague-Dawley rats (approximately 8 week-old; body weighed 180–220 g) from the Experimental Animal Center of Sichuan University (Chengdu, People's Republic of China). Animals were kept in a room where temperature (23 ± 1 °C), humidity ($55 \pm 5\%$) and lighting (light from 6 AM to 6 PM) were controlled.

2.5. Animal model for NMDA-induced neurotoxicity

NMDA-induced neurotoxicity was induced as described (Ma et al., 2010). Briefly, rats were anesthetized with an intra-peritoneal injection of 10% chloral hydrate (3 ml/kg body weight; KELONG, Chengdu, China). After the pupils were dilated with 1% tropicamide (Santen, Japan), a topical drop of 0.4% oxybuprocaine HCl (Santen, Japan) was applied to the cornea. Rat eyes were injected intravitreally with a 5- μ L bolus of (a) sterile 0.1 M PBS (pH 7.4) as control or (b) 20 nmol NMDA (Sigma-Aldrich, USA) in PBS, or (c) 15 μ g C3 + 20 nmol NMDA. NMDA is chemically stable, but we did not specifically ascertain (e.g. by NMR or HPLC) whether there is chemical or physical interaction between C3 and NMDA. Hamilton 10 μ L micro-syringes attached to a 30-G needle were used for intravitreal injections under an ophthalmic surgical microscope. Care was taken so as not to injure the lens and retina. At various time points after injection (18 h, 24 h, 48 h, 72 h), rats were sacrificed by an overdose intra-peritoneal injection of 10% chloral hydrate and the eyes were enucleated. Eyes with post-surgical complications such as cataract and intraocular hemorrhage (~2%) were excluded from the studies.

2.6. Preparation of retina cross-section and measurement of thickness of inner plexiform layer (IPL)

To investigate C3's effect on NMDA induced morphological changes, IPL thickness was assessed through HE staining (Lam et al., 1999). Eyes were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight at 4 °C and rinsed with running water for over 16 h, followed by dehydration and paraffin embedding. Transverse 7 μ m thick cross-sections through the optic disc were collected. The sections were de-waxed, re-hydrated, and stained with hematoxylin/eosin (HE). Images of each section were acquired at 100 $\times$ magnification under a light microscope for morphological analysis. Thickness measurements of the IPL were performed at a region about 1.5 mm from the center of the optic nerve head. Three sections were randomly chosen for each retina and five images were randomly taken for each section. The 15 images were measured using an image analyser (Image-Pro Plus Version 4.5.0.19, Media Cybernetics Inc., USA) and a mean value for each retina was calculated. All quantifications were performed in a masked manner. Three animals were used for each time point of each test condition.

2.7. Retina flat-mounts

Eyes were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 2 h at room temperature. After washing in 0.1 M PBS twice, 20 min each time, the anterior segments were removed. The retinas were dissected from the sclera and placed on a 1% gelatin-subbed glass slide with the nerve fiber layer (NFL) facing up, then flattened by four short cuts around the border. The retinas were briefly brushed with a fine brush to remove attached vitreous body and air-dried at room temperature.

2.8. Cresyl violet staining and cell counting

Retina flat-mounts were treated with 0.5% Triton X-100 for 1 min and stained with 0.5% cresyl violet for 10 min. After staining, each retina was rinsed in distilled water and dehydrated through an ascending alcohol series, cleared in xylene and coverslipped with Canada balsam.

Images were acquired with a light microscope (Eclipse TE2000-U, Nikon Corp., Japan). Each retina was divided into 4 quadrants –superior-temporal, inferior-temporal, superior-nasal, and inferior-nasal. Each quadrant was further divided into 3 regions – central, intermediate, and peripheral. Three images from each region were acquired at 400× magnification. The numbers of cresyl violet-stained neurons in the 36 images (distinguished from other cells morphologically by virtue of their larger size) were counted by two researchers in a masked manner. The counts were averaged and mean values expressed as number of cells per mm² for each retina was reported. Nissl stain was used to evaluate the C3-induced cell (including RGC) survival. When counting the cells, we attempted to distinguish RGCs from glial cells and vascular endothelial cells, but were not capable of distinguishing amacrine cells or other neuron-like cells from RGCs. Three animals were used for each time point of each test condition.

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Retina flat-mounts were hydrated with PBS and permeabilized with 10 µg/ml proteinase K (Sigma–Aldrich, USA) at 37 °C for 30 min. TUNEL staining was conducted according to the procedure described by the manufacturer in the *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany). Fluorescent images were captured under the IX-71 fluorescence microscope at 200× magnification.

2.10. Western blot

Retinas were dissected from eye cups, homogenized with lysis buffer (2% SDS in PBS with cocktail protease inhibitor, Sigma–Aldrich Chemie GmbH, Switzerland), and centrifuged at maximum speed (14,000 rpm) for 20 min at 4 °C. Protein concentrations were determined using Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, USA). Protein samples (15 µg per lane) were separated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Billerica MA, USA). The membranes were blocked with Tris-buffered saline (TBS) with 0.1% Tween-20 and 5% skim milk and incubated with anti-RhoA antibody (Santa Cruz, CA, USA) or anti-GAPDH antibody (ZSGB-BIO, Beijing, China), and then with peroxidase-labeled anti-rabbit IgG antibody or anti-mouse IgG antibody (ZSGB-BIO, Beijing, China). The immune complex was visualized under a FluorChem Chemiluminescent Imaging System (Alpha Innotech, San Leandro, CA, USA).

2.11. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Differences among groups were analyzed by One-Way ANOVA followed by Bonferroni tests (SPSS 15.0 statistic analysis software for Window, SPSS Inc., Chicago, IL, USA). Results with *P* values less than 0.05 (one-tailed) were considered statistically significant.

3. Results

3.1. C3 preparation and verification

C3 protein isolated and purified from C3-vector transformed *E. coli* was soluble and present in the cell supernatant after induction. The final concentration of C3 was 15 µg/µl after enrichment and purification. The purified C3 was detected as a single band of 26 kDa on SDS-PAGE as predicted (Fig. 1A).

C3-treated NIH 3T3 cells exhibited pronounced alteration in morphology, showing disrupted or absent actin cytoskeleton, relative to the untreated group (Fig. 1B). This confirmed the functionality of C3 (Liu et al., 2005; Udagawa and McIntyre, 1996), and also, proved that the C3 preparation was effective.

3.2. Cellular uptake of C3

Immunofluorescence was performed in NIH 3T3 cells treated with C3 to verify the cellular uptake of the protein. After 12 h treatment, C3 was present in the cytosol of the cells (Fig. 2), indicating that C3 was able to enter the cells.

3.3. Effects of C3 on IPL thickness

Intravitreal injection of NMDA caused IPL thinning in the rat retina. ($P < 0.0005$ at 24 h, 48 h, 72 h versus PBS group). Co-injection of C3 mitigated the changes (Fig. 3). At 18 h, IPL thickness was 43.4 ± 2.8 µm in the NMDA + C3-treated group, similar to 43.5 ± 3.8 µm in the NMDA group. IPL thickness in the NMDA group dropped to 29.2 ± 1.6 µm at 24 h and 30.1 ± 1.7 µm at 48 h. In the NMDA + C3 group, IPL thickness was 51.8 ± 1.2 µm at 24 h and 43.8 ± 0.8 µm at 48 h, significantly higher than their respective NMDA control group ($P < 0.0005$). At 72 h, IPL thickness was 31.9 ± 1.2 µm in the NMDA + C3 group, similar to 33.7 ± 1.4 µm in the NMDA group.

3.4. Effects of C3 on GCL cell survival

To investigate whether C3 enhanced the survival of GCL cells, cresyl violet staining was performed on flat-mount retinas 18 h, 24 h, 48 h and 72 h post intravitreal injection of NMDA with or without C3. The number of GCL cells in the PBS group was not significantly different between the four time points. As expected, NMDA decreased cell density in the GCL when compared to the PBS-treated group, but with no significant difference (Fig. 4). The possible reason for this is that not only RGC cells but also glial cells and other cells can be Nissl stained. The RGCs are not a high proportion of the total cells, which may explain why the NMDA-induced cell loss is not significant. At 24 h and 48 h, cell density in the NMDA group was 4997.8 ± 406.9 and 4348.5 ± 803.7 cells/mm², respectively. Compared with the NMDA group, the number of cells in the NMDA + C3 group was increased. Cell densities in the NMDA + C3 group at 24 h and 48 h were 6847.2 ± 788.4 and 6248.7 ± 32.9 cells/mm² respectively. The difference between these two time points was significant ($P < 0.05$).

3.5. Neuroprotective effects of C3 on NMDA-induced apoptosis in retina

To investigate the protective effects of C3 on NMDA-induced apoptosis, TUNEL on flat-mount retinas was performed 18 h, 24 h, 48 h and 72 h after intravitreal injection. As shown in Fig. 5, the PBS injection group did not have significant effects on cell apoptosis while the excitatory amino acid increased TUNEL-positive cells at all four time points after injection. The highest number of apoptotic cells was observed at the 24 h time point. Co-injection of C3 significantly decreased apoptotic cell number in the GCL compared with the NMDA-treated group. At 18 h, there were 541 ± 208 TUNEL positive cells/mm² in the NMDA + C3-treated group versus 2185 ± 1141 cells TUNEL positive/mm² in the NMDA group ($P < 0.05$). TUNEL positive cells in the NMDA + C3-treated group increased to 752 ± 299 cells/mm² at 24 h and 1363 ± 3 cells/mm² at 48 h. This was still significantly less than their respective NMDA control groups where TUNEL positive cells numbered 2910 ± 1279 cells/mm² at 24 h ($P < 0.05$) and 1939 ± 195 cells/mm² at 48 h ($P < 0.05$). However, the protective effect seemed to last for only 48 h. At 72 h, the numbers of apoptotic cells were similar in the two study groups (Fig. 5).

3.6. Effects of C3 on retina RhoA level

As C3 exoenzyme (C3) is a Rho inhibitor, RhoA levels in retina cells were examined by Western blot to ensure that the neuroprotective effect resulted from C3 uptake. Results showed an increase in the molecular weight of RhoA in cells treated with C3 at 18 h and 24 h, suggesting RhoA ribosylation in these cells (Morii and Narumiya, 1995) (Fig. 6). At the 72 h time point, RhoA had equal mobility in the NMDA-treated group and the NMDA + C3-treated group. Also, NMDA treatment increased RhoA expression at 48 h and 72 h, consistent with previous reports (Jeon et al., 2002; Kitaoka et al., 2004).

4. Discussion

The role of C3 in promoting RGC survival and axon regeneration has been shown *in vitro* and in optic nerve crush rat models (Fischer et al., 2004; Hu et al., 2007; Lehmann et al., 1999). However, in this *in vivo* study, C3 was evaluated in an NMDA excitotoxicity model. NMDA excitotoxicity has been putatively associated with glaucomatous retinopathy and was proposed to represent part of the underlying pathology of glaucoma.

Our results indicate that intravitreal injection of C3 protein can protect the retina against NMDA excitotoxicity for a short period of time, and Rho inhibition by C3 promotes neuronal survival against NMDA-induced neurotoxicity. These results, together with the IOP-lowering effects of C3 shown in previous studies (Liu et al., 2005), suggest that C3 or an analogue may be a useful approach for therapeutically targeting both the major causal risk factor in glaucomatous optic neuropathy and the neuropathy itself.

The precise mechanism by which C3 leads to neuroprotection is unclear. C3 is an inhibitor of Rho, which catalyzes the selective N-ADP-ribosylation of RhoA, RhoB, and RhoC of the Rho GTPase protein family and inhibits signaling pathways mediated by Rho GTPases (Vogelsgesang et al., 2007). Rho is an essential component in the excitotoxic cell death pathway. For example, RhoA itself is sufficient to induce the excitotoxic pathway via activation of p38 α (a stress-activated protein kinase that participates in a signaling cascade

controlling cellular responses to cytokines and stress) (Semenova et al., 2007). Therefore, it is reasonable to propose that C3 exerts its neuroprotective effects through downstream inhibition of Rho and deactivation of p38 α . C3/His-tag presence in RGCs and effects on ROCK downstream effectors would be a better proof of C3 *in vivo* permeability and functionality. Future studies will be needed to characterize the exact mechanism of this action.

It is interesting that the Rho family of proteins can be pro-apoptotic or anti-apoptotic, depending on cellular conditions and cell types (Kanekura et al., 2005). In myoblasts, inhibition of Rho by C3 causes apoptotic cell death *in vitro* (Reuveny et al., 2004). On the other hand, inactivation of Rho by C3 increases neuronal survival after spinal cord injury (Dubreuil et al., 2003); inhibition of Rho by H-1152P promotes neuronal survival in the rodent retina after optic nerve crush (Tura et al., 2009); and the ROCK inhibitors Fasudil and Y-27632 reduce RGC loss after NMDA-induced neurotoxicity (Kitaoka et al., 2004).

In conclusion, C3 is neuroprotective against NMDA-induced excitotoxicity in the retina. Combined with its IOP-lowering effects (Liu et al., 2005), it may suggest a novel approach in glaucoma treatment. However, the protective effect of C3 after a single intravitreal injection lasted less than 72 h in the current study. This short duration of action is likely a result of the relatively fast degradation of C3 in the vitreous. Regardless, it will limit the practical use of this protein as a therapeutic agent. Topical application may also be explored as a simplified delivery method. However, the relatively high molecular weight of C3 may make it difficult to cross the cornea. Furthermore, as excitotoxicity plays an important role in the progressive RGC death in glaucoma, other pathways may also be involved. Our results provide positive proof of concept of this approach. Future development and optimization of gene therapy, protein engineering, and/or sustained-release devices or formulations may allow the clinical application of C3 or related molecules for the treatment of glaucoma.

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Fig. 1.

Preparation and verification of C3. (A) IPTG treatment for 5 h induced C3 expression in *E. coli*. Purification of C3 was monitored by running samples from each stage on SDS-PAGE. PAGE gels were stained by Coomassie brilliant blue G-250. Purified C3 was shown as a single band on SDS-PAGE. (B) Phalloidin staining of C3-treated NIH 3T3 cells. C3-treated NIH 3T3 cells (30 $\mu\text{g}/\text{ml}$ for 12 h) exhibited pronounced alteration in morphology, showing slender processes, increased peripheral membrane ruffling, and disappearance of stress fibers, consistent with the inhibition of RhoA by C3 exoenzyme. Scale bars represent 100 μm (left panel) and 25 μm (right panel).

Fig. 2. Intracellular C3 visualized by DSU confocal fluorescence microscopy. NIH 3T3 cells were treated with 30 $\mu\text{g/ml}$ C3 for 12 h and labeled with anti-his antibody to visualize His-tagged C3. Intracellular C3 had a punctate appearance in most cells. Scale bar = 25 μm .

Fig. 3. C3 mitigation of NMDA-induced IPL thinning. (A) Representative images of H&E staining of rat retina cross-sections at various time points after intravitreal injection of 5 μ l PBS, 20 nmol NMDA, or 20 nmol NMDA+15 μ g C3. Scale bar = 50 μ m. (B) Quantitative analysis of IPL thickness. IPL thickness in the NMDA group was 43.5 ± 3.8 μ m at 18 h, 29.2 ± 1.6 μ m at 24 h, 30.1 ± 1.7 μ m at 48 h and 33.7 ± 1.4 μ m at 72 h. In the NMDA + C3 group, it was 43.4 ± 2.8 μ m at 18 h, 51.8 ± 1.2 μ m at 24 h, 43.8 ± 0.8 μ m at 48 h and 31.9 ± 1.2 μ m at 72 h. Significant reduction of the thickness of IPL was observed in the NMDA-treated group at 24 h, 48 h, 72 h. ($n = 3$; $***P < 0.0005$, NMDA-treated group versus the PBS group). Thinning of the IPL induced by NMDA injection was significantly mitigated in the NMDA + C3-treated group at 24 h and 48 h. ($n = 3$; $***P < 0.0005$, NMDA + C3-treated group versus the NMDA-treated group).

Fig. 4.

NMDA-induced loss of neurons in GCL is prevented by C3. (A) Representative images of cresyl violet-stained rat retina flat-mounts at the indicated time points after intravitreal injection of 5 μ l PBS, 20 nmol NMDA or 20 nmol NMDA+ 15 μ g C3. Scale bar = 25 μ m. (B) Quantitative analysis of cresyl violet staining data. Cell density in the NMDA group was 5221.0 ± 790.8 cells/mm² at 18 h, 4997.8 ± 406.9 cells/mm² at 24 h, 4348.5 ± 803.7 cells/mm² at 48 h and 5092.7 ± 715.5 cells/mm² at 72 h. In the NMDA + C3 group, it was 6583.0 ± 608.5 cells/mm² at 18 h, 6847.2 ± 788.4 cells/mm² at 24 h, 6248.7 ± 32.9 cells/mm² at 48 h and 6248.7 ± 750.8 cells/mm² at 72 h. Cell numbers in the GCL were decreased at all time points following NMDA treatment, but with no significant difference. Co-injection of C3 significantly attenuated the reduction in cell counts at 24 h and 48 h, compared with the NMDA group. ($n = 3$; $*P < 0.05$, NMDA + C3-treated group versus the NMDA group).

Fig. 5.

NMDA-induced apoptosis of GCL neurons is prevented by C3. (A) Representative images of TUNEL-labeled rat retina flat-mounts at the indicated time points after intravitreal injection of 5 μ l PBS, 20 nmol NMDA or 20 nmol NMDA +15 μ g C3. Scale bar = 100 μ m. (B) Quantitative analysis of TUNEL-stained cells. The number of TUNEL-positive cells in the NMDA group was $2185 \pm 1141/\text{mm}^2$ at 18 h, $2910 \pm 1279/\text{mm}^2$ at 24 h, $1939 \pm 195/\text{mm}^2$ at 48 h and $1633 \pm 33/\text{mm}^2$ at 72 h. In the NMDA + C3 group, it was $541 \pm 208/\text{mm}^2$ at 18 h, $752 \pm 299/\text{mm}^2$ at 24 h, $1363 \pm 3/\text{mm}^2$ at 48 h and $1523 \pm 72/\text{mm}^2$ at 72 h. NMDA significantly induced apoptosis of cells within the GCL. (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, NMDA-treated group versus the PBS group). The NMDA + C3-treated group had significantly fewer TUNEL-positive cells at 18 h, 24 h, 48 h. (* $P < 0.05$, NMDA + C3-treated group versus the NMDA group). There were almost no TUNEL-positive cells in PBS or C3 only treated groups (data not shown).

Fig. 6.

Effect of NMDA and C3 intravitreal injections on retina RhoA ribosylation. C3 treatment changed the mobility of RhoA in SDS-PAGE, indicative of ribosylation. Compared with the NMDA-treated group, RhoA in the NMDA + C3-treated group showed higher molecular weight at 18 h and 24 h, but not at 72 h. ($n = 4$).