NEURAL REGENERATION RESEARCH
April 2014, Volume 9, Issue 8
www.nrronline.org

Upregulation of Nogo receptor expression induces apoptosis of retinal ganglion cells in diabetic rats

Xuezheng Liu[1], Zhongfu Zuo[1], Wanpeng Liu[1], Zhiyun Wang[1], Yang Hou[2], Yunjie Fu[1], Yuzhi Han[1]

1 Department of Anatomy, Liaoning Medical University, Jinzhou, Liaoning Province, China
2 Scientific Experimental Center, Liaoning Medical University, Jinzhou, Liaoning Province, China

Xuezheng Liu and Zhongfu Zuo contributed equally to this study.

Corresponding author:
Xuezheng Liu, M.D., Department of Anatomy, Liaoning Medical University, Jinzhou 121001, Liaoning Province, China, Liuxuezheng168@vip.sina.com.

doi:*10.4103/1673-5374.131597*
http:*//www.nrronline.org/*

Accepted: *2014-03-28*

Abstract

The Nogo receptor is an essential factor for neuronal apoptosis, but the changes in Nogo receptor expression in the retina and the effects of the Nogo receptor on retinal ganglion cell apoptosis in diabetes mellitus remain unclear. We found that Nogo receptor expression was mainly visible in retinal ganglion cells of a rat model of diabetes mellitus induced by streptozotocin. At 12 weeks after onset of diabetes mellitus, Nogo receptor and Rho kinase expression significantly increased in the retina, and retinal ganglion cell apoptosis was apparent. When RNA interference was used to suppress Nogo receptor expression in rat retina, Rho kinase expression was obviously inhibited, and retinal ganglion cell apoptosis was evidently reduced in rats with diabetes mellitus. These results indicate that upregulation of Nogo receptor expression is an important mechanism of retinal ganglion cell apoptosis in rats with diabetes mellitus.

Key Words: *nerve regeneration; diabetes mellitus; diabetic retinopathy; visual acuity; retinal ganglion cells; apoptosis; Nogo receptor; Rho kinase; myelin-associated protein; NSFC grant; neural regeneration*

Funding: *This study was supported by the National Natural Science Foundation of China, No. 31140072, 81300931; the Liaoning Provincial Department of Science and Technology Project, No. 2011225015; the Natural Science Foundation of Liaoning Province in China, No. 2013022055.*

Liu XZ, Zuo ZF, Liu WP, Wang ZY, Hou Y, Fu YJ, Han YZ. Upregulation of Nogo receptor expression induces apoptosis of retinal ganglion cells in diabetic rats. Neural Regen Res. 2014;9(8):815-820.

Introduction

Diabetic retinopathy is a key complication of diabetes mellitus, but its pathogenesis remains unclear. Diabetic retinopathy lacks effective therapies and has been a key factor for blindness in adults (Silva et al., 2010). Nervous system lesions in the retina are a key reason for diabetic retinopathy and a main mechanism for diminution of vision (Robinson et al., 2012). Retinal ganglion cell axons are a major component of optic nerves. The injury, structural changes, hypofunction and apoptosis of retinal ganglion cells are major factors for decreased vision in patients with diabetic retinopathy (Lieth et al., 2000; Kim et al., 2003), but the mechanisms underlying retinal ganglion cell apoptosis remain poorly understood in patients with diabetes mellitus.

Nogo receptor (NgR) is a membrane receptor associated with neural regeneration, and can be combined with myelin-associated proteins, such as Nogo protein, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein (Li et al., 2004). Activated NgR can suppress neural regeneration, leading to neuronal atrophy and apoptosis (Peng et al., 2010; Pernet et al., 2012). NgR is extensively expressed in the nervous system, but can only be observed in retinal ganglion cells of the retina (Yin et al., 2007). Upregulation of NgR expression could induce retinal ganglion cell apoptosis and diminution of vision after occurrence of glaucoma (Fu et al., 2011). Rho-associated protein kinase (ROCK), an NgR downstream molecule, was strongly associated with neuronal apoptosis (Tan et al., 2010; Wälchli et al., 2013). Retinal ganglion cell apoptosis is a crucial mechanism of diabetic retinopathy, but it remains unclear whether retinal ganglion cell apoptosis is associated with the alterations in NgR gene expression after diabetic retinopathy. We still do not know which molecular mechanisms are involved when NgR participates in retinal ganglion cell apoptosis. Therefore, this study sought to explore the changes in NgR expression in the diabetic retina, to investigate the effects and mechanisms of NgR in the occurrence and development of diabetic retinopathy, and to provide new ideas for clinical treatment of diabetic retinopathy.

Materials and Methods

Animals

A total of 40 clean adult Sprague-Dawley rats aged 3 months and weighing 220–250 g were enrolled in this study (Animal license No. SCXK (Liao) 20030007). Rats obtained from Liaoning Medical University in China were housed under controlled temperature and humidity with a 12-hour light/dark cycle (light on at 6:00 and off at 18:00). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the protocol was approved by the Animal Care Committee from Liaoning

Medical University, China.

Grouping

A total of 40 rats were randomly assigned to control, diabetes mellitus, siNgR and siRNA blank groups. Rat models of diabetes mellitus were induced by streptozotocin in the diabetes mellitus, siNgR and siRNA blank groups. Lentivirus-packed NgR antisense nucleotide sequence (10 μL) was injected into bilateral vitreous bodies of rats in the siNgR group. Lentivirus-packed silenced nucleotide sequence (10 μL) was administered in bilateral vitreous bodies of rats in the siRNA blank group. Preservation solution (10 μL), *i.e.*, PBS supplemented with 10% glycerol, was injected into bilateral vitreous bodies of rats in the diabetes mellitus and control groups. 12 weeks later, morphological and molecular biological indexes were detected in each group.

Preparation of lentivirus-packed nucleotide sequence

The siNgR antisense oligonucleotide sequence was designed in accordance with a previous study (Fry et al., 2007). Nucleotide sequence was synthesized and lentivirus package was produced by Shanghai GenePharma Co., Ltd. (Shanghai, China). siNgR antisense oligonucleotide sequence was 5′-AAT GAC TCT CCA TTT GGG ACT-3′, whilst silenced nucleotide sequence was 5′-UUC UCC GAA CGU GUC ACG UTT-3′. Virus titer was 1×10^{12} TU/mL.

Establishment of an animal model of diabetes mellitus

In accordance with a previously published method (Zuo et al., 2011), an animal model of diabetes mellitus was established. After 12-hour fasting, rats were intraperitoneally injected with streptozotocin 60 mg/kg (dissolved in pH 4.5 0.1 mol/L citrate buffer solution) for 48 hours. Blood was collected from the tail tip. Blood glucose concentration was measured using a Blood Glucose Meter (Roche Diagnostics, Mannheim, Germany). Blood glucose concentration > 16.7 mmol/L served as a marker for successful establishment of the model. Rats with blood glucose lower than the above-mentioned criteria were excluded. Rats from the control group were intraperitoneally injected with citrate buffer solution without streptozotocin.

Preparation of retinal specimens

At 12 weeks after model induction, rats were intraperitoneally injected with 4% sodium pentobarbital (60 mg/kg). After opening the thoracic cavity, a perfusion cannula was inserted through the cardiac apex to the root of the ascending aorta. Blood was flushed away with 100 mL of physiological saline, and tissue was fixed with 4% paraformaldehyde (pH 7.3, 500 mL) for 2 hours. The eyes were removed and the anterior segment and vitreous body were peeled away. The retina was dehydrated with ethanol, permeabilized with xylene, and sliced into 8 μm paraffin sections.

Double-label fluorescent immunohistochemistry for NgR and Brn3a expression in the rat retina

Sections were dewaxed and hydrated. Antibodies were diluted by diluent, containing 0.01 mol/L PBS, 0.1% Triton X-100 and 10% normal goat serum. Rabbit anti-rat NgR (1:200) was mixed with mouse anti-rat Brn3a (1:200; Sigma, St. Louis, MO, USA). Sections were incubated with the mixture at room temperature for 2 hours, then at 4°C overnight, followed by PBS washes (10 minutes × 3). Sections were then incubated with a mixture containing Alexa 488-labeled donkey anti-rabbit IgG and Alexa 594-labeled donkey anti-mouse IgG (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) for 4 hours at room temperature, followed by PBS washes (10 minutes × 3). These sections were mounted, observed and photographed with a fluorescence microscope (CX31-32RFL; Olympus, Tokyo, Japan).

Fluorescence immunohistochemical detection for ROCK expression in the rat retina

Retinal sections were dewaxed, hydrated, and then incubated with 10% normal fetal bovine serum for 30 minutes at room temperature. This incubation was followed by exposure to mouse anti-rat ROCK (1:200; Sigma) at room temperature for 4 hours, and at 4°C overnight. After washes with PBS (10 minutes × 3), these sections were incubated with A488-labeled goat anti-mouse IgG (1:500; Sigma) for 4 hours at room temperature. Sections were then washed again and incubated with DAPI (1:1,000; Sigma) for 15 minutes, mounted, blocked, and observed with an inverted fluorescence microscope (Olympus). Four fields of each retina were randomly observed at 400 × magnification. The numbers of ROCK-positive cells and retinal ganglion cells were counted. Each field was read three times, and the mean value was obtained. The proportion of positive cells stained for ROCK in retinal ganglion cells was calculated.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for detection of apoptosis of retinal ganglion cells in the rat retina

TUNEL assay was used to detect the apoptosis of retinal ganglion cells in the rat retina (Jiang et al., 2011). Sections were dewaxed, hydrated and washed with PBS (3 × 10 minutes). Procedures were conducted in accordance with the instructions on the TUNEL kit (KeyGEN BioTECH, Nanjing, Jiangsu Province, China). Sections were visualized with 3, 3′-diaminobenzidine, washed with water for 30 minutes, and counterstained with hematoxylin for 5 minutes. Subsequently, sections were differentiated with differentiation medium for 3 seconds, washed for 15 minutes, permeabilized with xylene, and dehydrated through a graded alcohol series. Sections were finally mounted with neutral resin, and observed with an inverted microscope. Four fields of each retina were randomly read at 200 × magnification. The numbers of TUNEL-positive cells and retinal ganglion cells were counted. Each field was counted three times, and the mean value was obtained. The proportion of positive TUNEL stained retinal ganglion cells was calculated.

Western blot assay for detection of NgR and ROCK protein expression in the rat retina

At 12 weeks after model induction, rats were intraperitoneally injected with 4% sodium pentobarbital (60 mg/kg). Eye-

Table 1 Effects of intravitreal injection of siNgR nucleotide sequence on blood glucose (mmol/L) and body weight (g) of rats with diabetes mellitus

Group	Blood glucose		Body weight	
	Pre-induction	12 weeks post-induction	Pre-induction	12 weeks post-induction
Control	4.5±0.4	4.5±0.4	231.2±4.8	503.2±10.7
Diabetes mellitus	4.8±0.5	17.2±1.2*	239.1±5.3	300.4±7.5*
siRNA blank	4.9±0.4	20.4±1.5*	228.6±6.3	310.6±8.3*
siNgR	4.7±0.3	18.7±2.0*	235.5±7.1	315.7±9.2*

Data are expressed as mean ± SEM, with ten rats in each group. One-way analysis of variance and least significant difference test were used. *$P < 0.01$, *vs.* control group.

balls were obtained and retinas were removed. Samples were treated with lysate (Roche, Basal, Switzerland), triturated on ice, and centrifuged at 10,000 × *g* at 4°C for 20 minutes (Shanghai Anting Scientific Instrument Factory, Shanghai, China). Protein content of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Protein was isolated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The polyvinylidene difluoride membrane was blocked with Tris-buffered saline containing Tween-20 at 37°C for 90 minutes (containing 50 g/L skimmed milk powder). The sections were incubated with primary antibodies (goat anti-rat NgR antibody, 1:1,000, Sigma; goat anti-rat ROCK antibody, 1:1,000, Santa Cruz Biotechnology; goat anti-rat β-actin antibody, 1:1,000, Santa Cruz Biotechnology) at 4°C overnight. After three washes with Tris-buffered saline containing Tween-20, each for 10 minutes, sections were incubated with horseradish peroxidase-labeled donkey anti-goat IgG (1:1,000; Santa Cruz Biotechnology) for 2 hours at room temperature. The samples were visualized with western blot substrate enhanced chemiluminescence kit (Abcam, Cambridge, UK). Absorbance of each band was scanned with a gel imaging system (Ultra-Violet Products Ltd., Cambridge, UK). Standardization to the expression of β-actin was then performed.

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA), and expressed as mean ± SEM. One-way analysis of variance was performed using a randomized block design. Paired comparison was completed using least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

Intravitreal injection of siNgR nucleotide sequence did not significantly affect blood glucose and body weight in rats with diabetes mellitus

Before model induction, no significant difference in blood glucose concentration or body weight was detected in rats from each group ($P > 0.05$). At 12 weeks after model establishment, body weight was slightly heavier and blood glucose levels were higher compared with the control group ($P < 0.01$). Transfection with antisense nucleotide did not influence rat body weight and blood glucose ($P > 0.05$; Table 1).

siNgR inhibited NgR and ROCK expression in the retina

Results of double-label fluorescent immunohistochemistry demonstrated that a large amount of NgR expression was detected in the ganglion cell layer of rat retina in the control group, and coexisted with Brn3a (Figure 1). Brn3a is a marker of retinal ganglion cells. The coexistence of NgR and Brn3a indicated that NgR is expressed in retinal ganglion cells and provides morphological evidence for NgR expression interfering and affecting apoptosis in retinal ganglion cells.

Western blot assay results exhibited that at 12 weeks after model establishment, NgR and ROCK expression were higher in the diabetes mellitus and siRNA blank groups compared with the control group ($P < 0.01$). No significant difference in NgR and ROCK expression was detectable in the siNgR group ($P > 0.05$; Figure 2).

Inhibition of NgR expression suppressed ROCK expression in retinal ganglion cells

Immunohistochemical results revealed that 9.1±0.5% of cells were positively stained for ROCK in retinal ganglion cells from rats in the control group. At 12 weeks after model establishment, the proportion of ROCK stained cells was significantly higher in the diabetes mellitus and siRNA blank groups 35.2±1.7%, 30.6±2.1%, respectively than in the control group); $P < 0.01$. The number of ROCK-positive retinal ganglion cells was significantly lower in the siNgR group than that in the diabetes mellitus group (11.6±0.7%, $P < 0.05$). These results suggested that the inhibition of NgR expression could apparently downregulate ROCK protein expression in retinal ganglion cells of rats with diabetes mellitus (Figure 3).

Downregulation of NgR expression suppressed apoptosis in retinal ganglion cells in rats with diabetes mellitus

TUNEL-positive neurons were observed in the rat retina of the control group. At 12 weeks, a large number of TUNEL-positive retinal ganglion cells were detected in the diabetes mellitus, siRNA blank and siNgR groups, respectively (46.8±3.5%, 51.3±2.1%, and 18.7±1.5%; Figure 4). The proportion of TUNEL-positive cells in retinal ganglion was significantly lower in the siNgR group compared with

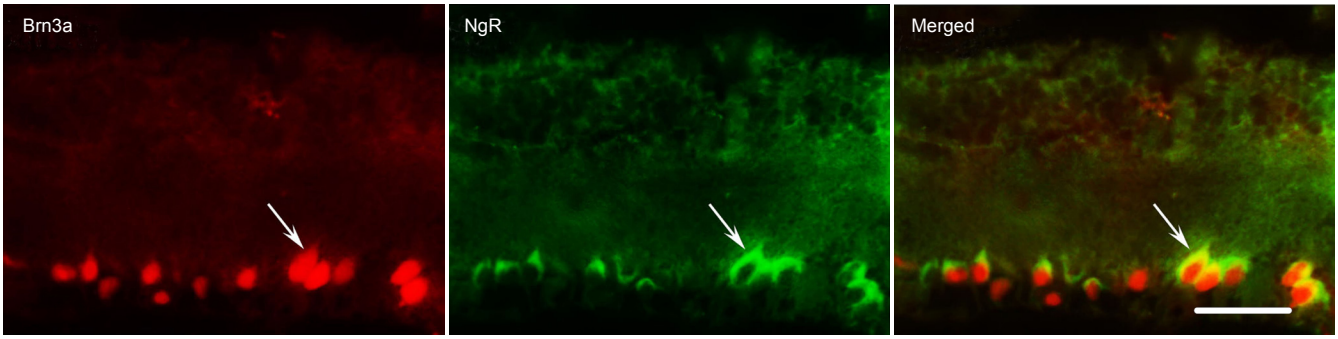

Figure 1 Coexistence of Brn3a and NgR in retinal ganglion cells (double-label fluorescent immunohistochemistry).
Brn3a expression (red, Alexa 594 fluorescent label, arrow) and NgR expression (green, Alexa 488 fluorescent label, arrow) coexisted in retinal ganglion cells (yellow, arrow). NgR: Nogo receptor. Scale bar: 10 μm.

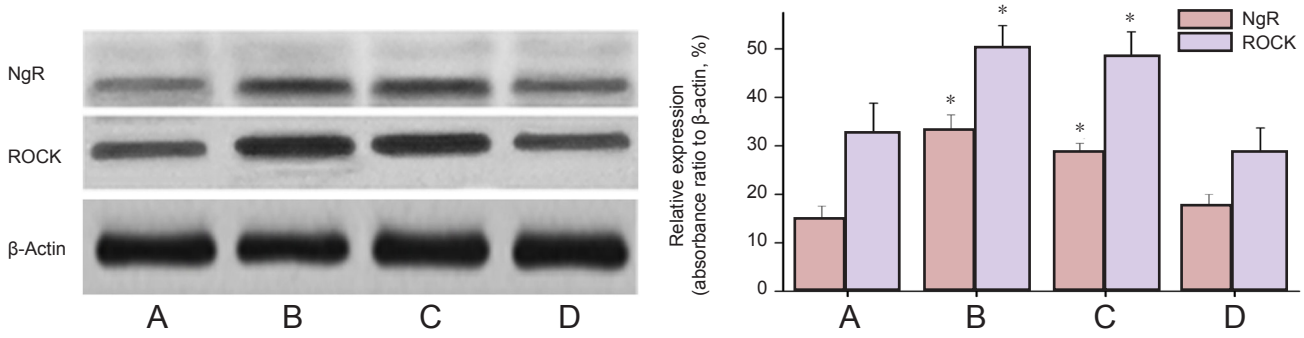

Figure 2 Western blot assay for siNgR effects on NgR and ROCK protein expression in the retina of rats with diabetes mellitus.
At 12 weeks after model establishment, NgR and ROCK protein expression levels were significantly higher in the diabetes mellitus and siRNA blank groups than those in the control group. No significant difference in NgR and ROCK protein expression was visible in the siNgR group. The data are expressed as mean ± SEM. One-way analysis of variance and least significant difference test were used. $*P < 0.01$, *vs.* control group. A: Control group; B: diabetes mellitus group; C: siRNA blank group; D: siNgR group. NgR: Nogo receptor.

the diabetes mellitus group ($P < 0.01$). Results indicated that inhibition of NgR expression could evidently suppress apoptosis of retinal ganglion cells in the rat retina.

Discussion

Pathological changes in neurons directly cause a reduction in visual acuity in patients with diabetic retinopathy. Electroretinogram, contrast of visual acuity, range of visual field and color sense all showed obvious abnormalities in these patients (Lieth et al., 2000; Zhang et al., 2013). Retinal ganglion cell axons are a major part of the optic nerve. Injured retinal ganglion cells are difficult to regenerate, and retinal ganglion cell injury is a key factor for the decrease in visual acuity of diabetic retinopathy patients (Fischer et al., 2004; Harvey et al. 2006). This study also found that nervous system lesions in retina are an important pathological alteration in diabetic retinopathy. The number of TUNEL-positive retinal ganglion cells was apparently increased in rats with diabetes mellitus for 3 months, with the presence of apoptosis. The mechanisms of retinal ganglion cell apoptosis in the diabetic retina were not clear. Excitatory neurotransmitters such as glutamate, branched-chain amino acids and homocysteine, accumulated in the diabetic retina (Ola et al., 2013). The lacks of folic acid and vitamin B12 are important mechanisms in the apoptosis of retinal ganglion cells of the diabetic retina (Ola et al., 2013). Abnormal retinal metabolism is the key reason for the apoptosis of retinal ganglion cells, but the mechanisms underlying the apoptosis of retinal ganglion cells in the diabetic retina remain unclear. NgR is a myelin-associated protein, and can bind to Nogo protein, myelin-associated glucoprotein and oligodendrocyte myelin glycoprotein (Li et al., 2004). Activated NgR can suppress neural regeneration and promote neuronal apoptosis (Peng et al., 2010). NgR extensively expresses in nervous system, but only can be observed in retinal ganglion cells of the retina (Yin et al., 2007). The apoptosis of retinal ganglion cells is an essential mechanism of diabetic retinopathy, and strongly associated with NgR (Kern et al., 2008; Fu et al., 2011). To explore the effects and mechanisms of NgR in the apoptosis of retinal ganglion cells of diabetic retina, the present study detected the coexistence of NgR and Brn3a in the retina by double-label fluorescent immunohistochemistry (Badea and Nathans, 2011), and confirmed that NgR was only expressed in retinal ganglion cells. To investigate the effects of NgR on retinal ganglion cells of rats with diabetes mellitus, antisense oligonucleotide specificity was used to inhibit NgR expression in the diabetic retina. The downregulation of NgR expression in the diabetic retina evidently suppressed the apoptosis of retinal ganglion cells of rats with diabetes mellitus. Results indicated that the upregulation of NgR

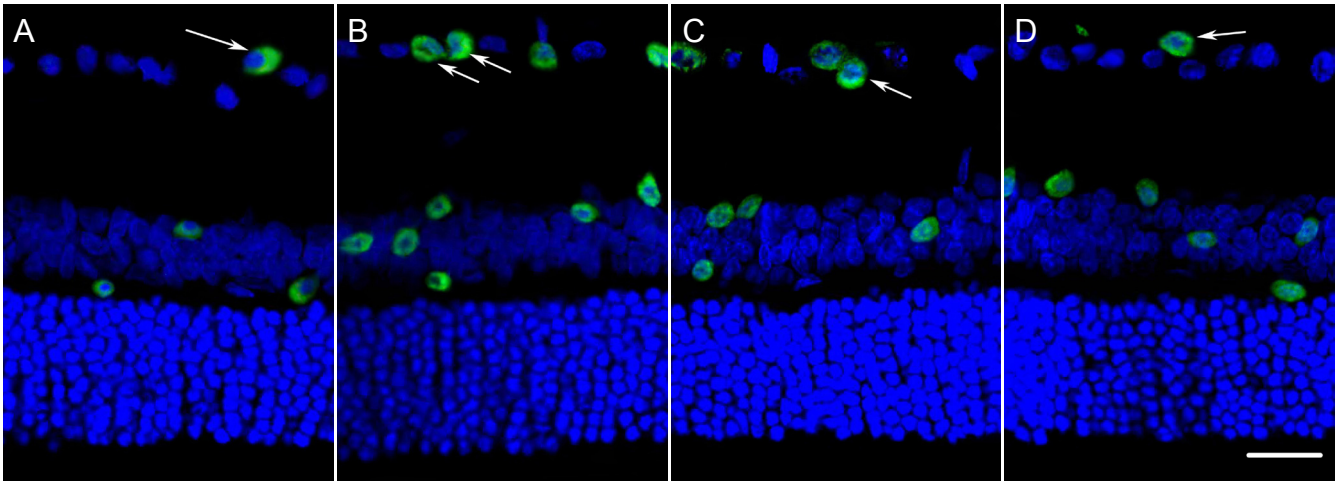

Figure 3 Inhibition of NgR expression affected the expression of ROCK in retinal ganglion cells from rat retina (immunohistochemical staining).
Compared with the control group (A), at 12 weeks, the numbers of ROCK-positive retinal ganglion cells were significantly higher in the diabetes mellitus group (B) and siRNA blank group (C). Compared with the diabetes mellitus group, the number of ROCK-positive retinal ganglion cells was significantly lower in the siNgR group (D). Arrows show ROCK-positive retinal ganglion cells. Green: Alexa 488 fluorescent label. NgR: Nogo receptor; Rock: Rho-associated protein kinase. Scale bar: 10 μm.

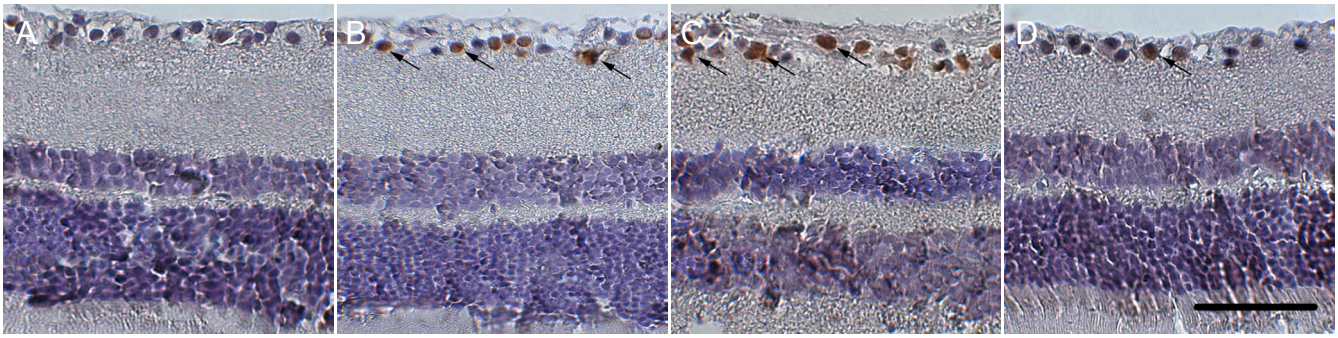

Figure 4 Downregulation of NgR expression suppressed apoptosis of retinal ganglion cells in rat retina (TUNEL staining, × 200).
TUNEL-positive retinal ganglion cells were not detectable in the control group (A). At 12 weeks after model establishment, TUNEL-positive retinal ganglion cells were visible in the diabetes mellitus group (B) and siRNA blank group (C). The number of TUNEL-positive retinal ganglion cells was noticeably lower in the siNgR group (D) than that in the diabetes mellitus group. Arrows exhibit TUNEL-positive retinal ganglion cells in the retina. NgR: Nogo receptor; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling. Scale bar: 20 μm.

expression was a key mechanism of the apoptosis in retinal ganglion cells from diabetic rats. A previous study showed that NgR expression apparently increased in retinal ganglion cells after glaucoma occurrence, so to inhibit NgR expression could suppress the apoptosis of retinal ganglion cells (Fu et al., 2011). These findings were similar to results seen in this study. Therefore, increased NgR expression is an important mechanism underlying the apoptosis of retinal ganglion cells.

ROCK, a serine-threonine kinase and an NgR downstream molecule, is strongly associated with retinal ganglion cell apoptosis. ROCK protein inhibitor Y-27632 promoted the regeneration of retinal ganglion cell axons, inhibited retinal ganglion cell apoptosis and contributed to neural regeneration in rats receiving optic neurotomy (Lingor et al., 2007; Lingor et al., 2008). Inhibiting NgR expression may downregulate ROCK protein expression in the retina of diabetic rats, but ROCK protein is extensively expressed in the retina (Li et al., 2013). To analyze the effects of downregulating NgR protein expression on ROCK protein expression in retinal ganglion cells of diabetic rats, this study detected ROCK protein expression in retinal ganglion cells using immunohistochemical methods, and detected ROCK changes in the retina using western blot assay, which supported immunohistochemical results. Results demonstrated that NgR regulated ROCK expression in the retina of diabetic rats. A large number of apoptotic retinal ganglion cells were visible when ROCK expression was increased. Moreover, to inhibit NgR expression and to downregulate ROCK expression could noticeably suppress the apoptosis of retinal ganglion cells of diabetic rats. These results suggest that ROCK protein is a key molecular mechanism for the regulatory effect of NgR on the apoptosis of retinal ganglion cells of diabetic rats.

This study verified that increased NgR expression is a key factor for the apoptosis of retinal ganglion cells, and its mechanism is probably associated with the upregulation of NgR on ROCK protein expression. However, this study

has some limitations, and did not explore the downstream mechanism of the apoptosis of retinal ganglion cells of ROCK-induced diabetic retina. Moreover, NgR can induce apoptosis of retinal ganglion cells of diabetic rats through other pathways. As we have seen, there are no reports on NgR effects on the apoptosis of retinal ganglion cells of diabetic rats, or on the underlying mechanisms. This study investigated the effects and mechanisms of NgR on the apoptosis of retinal ganglion cells of diabetic rats, provided new ideas for revealing pathogenesis of diabetic retinopathy and clinical treatment of diabetic retinopathy.

Author contributions: *Liu XZ participated in study design and technical support. Zuo ZF participated in morphological study and wrote the manuscript. Liu WP was in charge of morphological study. Wang ZY participated in statistical analysis. Hou Y detected molecular biology. Fu YJ and Han YZ established animal models. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Peer review: *NgR has been paid great attention, due to its specific target molecule effects during signal transduction of myelin-inhibited axon regeneration. This study used animal models of diabetes mellitus, verified that NgR only expressed in retinal ganglion cells. NgR and ROCK expression significantly increased in retinal ganglion cells, and ganglion cell apoptosis was obvious. Moreover, to inhibit NgR expression could decrease the apoptosis of retinal ganglion cells of diabetic rats. Results confirmed that NgR extensively expressed in optic nerve and suppressed neural regeneration. This study provided an experimental basis for finding new blockers and drugs to promote optic nerve regeneration.*

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