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Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist inhibits apoptosis of retinal ganglion cells in a rabbit model of optic nerve injury★

Ruijia Wang, Xinping Luan, Yiti Mu, Hongyu Jia, Jingxuan Xu

Department of Neurosurgery, Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

Abstract

A rabbit model of traumatic optic nerve injury, established by occlusion of the optic nerve using a vascular clamp, was used to investigate the effects of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist GYKI 52466 on apoptosis of retinal ganglion cells following nerve injury. Hematoxylin-eosin staining and a terminal deoxynucleotidyl transferase dUTP nick end labeling assay showed that retinal ganglion cells gradually decreased with increasing time of optic nerve injury, while GYKI 52466 could inhibit this process. The results demonstrate that following acute optic nerve injury, apoptosis of retinal ganglion cells is a programmed process, which can be inhibited by the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist.

Key Words: optic nerve injury; retinal ganglion cells; glutamate; alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Abbreviations: RGCs, retinal ganglion cells; NMDA, N-methyl-D-aspartate; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Ruijia Wang★, Studying for master's degree, Department of Neurosurgery, Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

Corresponding author: Xinping Luan, Professor, Chief physician, Department of Neurosurgery, Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China luanxinping@yahoo.com.cn

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INTRODUCTION

Loss of vision in acute optic nerve injury results from optic nerve axonal injury and subsequent death of retinal ganglion cells (RGCs)[1-4]. Slowing or inhibiting secondary degeneration of RGCs following optic nerve injury can provide the basis for effective treatment of optic nerve injury, and can promote the recovery of visual function limited by the low regenerative capability of RGCs[5]. Recent reports have demonstrated that following optic nerve injury, altered microenvironment greatly affects the repair and regeneration of the optic nerve[6-9]. Additional studies have shown that following acute optic nerve injury and death of RGCs, the resulting elevated intravitreal glutamate levels are potentially neurotoxic in the microenvironment of RGCs[10-11]. Glutamate acts directly on N-methyl-D-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, indirectly stimulating the combined conduction of photoreceptor, bipolar cell, and retinal cell processes[12]. Wang *et al* [13] reported that the NMDA receptor antagonist dizocilpine can effectively reduce apoptosis of RGCs in a rabbit model of optic nerve injury. To investigate whether an AMPA receptor antagonist also exhibits anti-apoptotic effects on RGCs, we established a rabbit model of traumatic optic nerve injury, treated it with AMPA antagonist GYKI 52466, and observed changes in the number of RGCs following optic nerve injury.

RESULTS

Quantitative analysis of experimental animals

Eighty New Zealand rabbits were used as models of traumatic optic nerve injury. They were randomly divided into a treatment group and a control group, with 40 rabbits in each group. In the treatment group, GYKI 52466 was administered intraperitoneally, and in the control group, physiological saline was administered intraperitoneally. The right eyes served as model controls and the left eyes served as blank controls. At 1, 3, 7, 14, and 21 days after optic nerve injury, eight rabbits from each group were used for experimentation. All 80 rabbits were included in the final analysis.

Gross changes in operated eyes of rabbits with optic nerve injury

After induction of traumatic optic nerve injury,

incisions healed well, without inflammatory reaction, and corneas were transparent. No traumatic cataract, intravitreal inflammatory reaction, or hematocele was observed. Retinal blood supply was normal. There was no hemorrhage or retinal detachment. The pupils of the operated eyes dilated 2–4 mm and relative afferent pupillary defect occurred. All symptoms indicated success in induction of traumatic optic nerve injury, and traumatic optic nerve injury was achieved in all 80 rabbits.

GYKI 52466 reduced loss of RGCs in rabbits with traumatic optic nerve injury

Hematoxylin-eosin staining showed that at the early stage of traumatic optic nerve injury (1, 3 days), cells in the retinal cell layer and inner nuclear layer were arranged in a disorderly manner, chromatin was decentralized towards the periphery, nuclei were shrunken, cellular interspace was increased, cells were swollen, and RGCs decreased in number. At 7, 14, and 21 days after traumatic optic nerve injury induction, the above-mentioned changes progressed and the number of RGCs were further reduced. At 1, 3, 7, 14, and 21 days after injury, RGCs in each tissue area (25 μm × 25 μm) stained by hematoxylin-eosin were reduced by 28.32%, 37.96%, 39.27%, 56.72%, and 64.51%, respectively. These findings suggested that following acute optic nerve injury, apoptosis of RGCs is a continually programmed process. After GYKI 52466 administration, cell morphology in the retinal cell layer and inner nuclear layer were relatively normal, the degree of injury decreased, and the loss of RGCs was reduced (Figure 1, Table 1).

GYKI 52466 inhibited apoptosis of RGCs in rabbits with traumatic optic nerve injury

TUNEL staining showed that following optic nerve injury, most RGCs were stained, and at 3 days after injury, loss of RGCs and DNA fragmentation occurred. At 14 days after optic nerve injury, TUNEL-positive cells could be observed in the retinal cell layer. At 3 and 14 days after optic nerve injury, there were significantly fewer TUNEL-positive cells in the treatment group than in the model control group (Figure 2).

DISCUSSION

Following acute optic nerve injury, retinal ischemia causes injury of the retinal cell layer and inner nuclear layer, and cells in the retinal cell layer are greatly reduced[14]. We successfully established a rabbit model of traumatic optic nerve injury as confirmed by gross observation and pupillary reflex. Using this model, loss of RGCs began at the early stage of optic nerve injury and was characterized by *in situ* DNA fragments, nuclear chromatin marginalization, and morphological condensation. At 1, 3, 7, 14, and 21 days after acute optic nerve injury, RGCs were greatly reduced. Understanding of persistent apoptosis of RGCs would help further explain the mechanism of retinal ischemia[15].

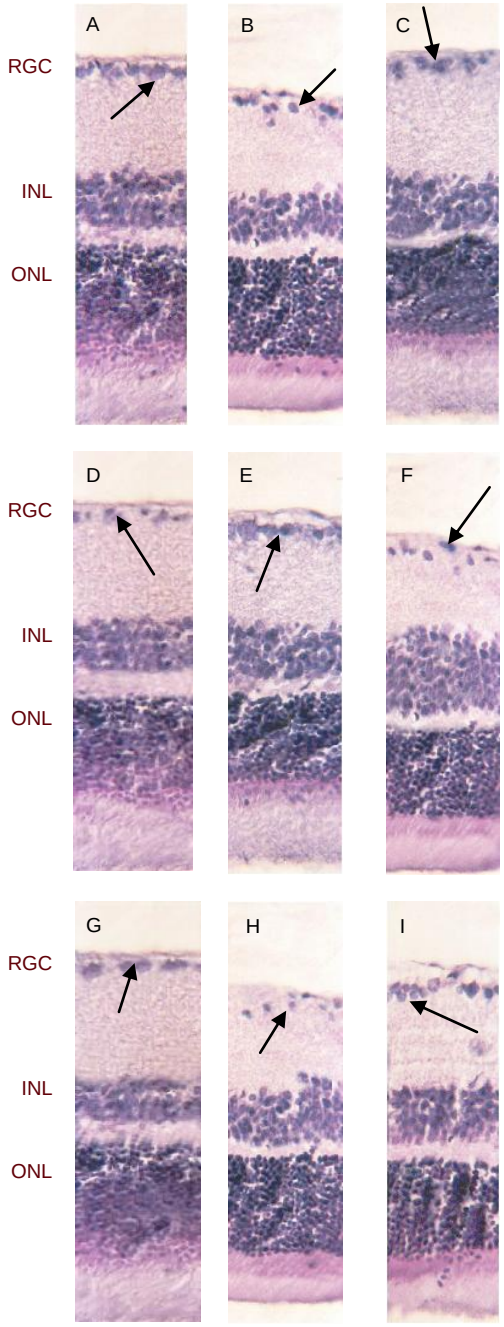

Figure 1 Morphology of rabbit RGCs after hematoxylin-eosin staining (optical microscope, × 400).

Loss of RGCs (arrows) increases with increasing time after optic nerve injury. Compared to corresponding time points in the model control group, loss of RGCs in the treatment group decreased, and retinal morphology is more normal. RGCs: Retinal ganglion cells; INL: inner nuclear layer; ONL: outer nuclear layer.

(A) Blank control group; (B) model control group at 3 days; (C) treatment group at 3 days; (D) model control group at 7 days; (E) treatment group at 7 days; (F) model control group at 14 days; (G) treatment group at 14 days; (H) model control group at 21 days; (I) treatment group at 21 days.

Table 1 Quantitation of rabbit retinal ganglion cells in each group (number of surviving retinal ganglion cells/ 25 μm × 25 μm)

Injury days	Blank control	Model control	Treatment
1	34.00±1.31	24.37±0.91[a]	29.75±2.49[b]
3	34.25±1.03	22.25±0.70[a]	25.50±2.27[b]
7	34.38±0.74	20.88±0.64[a]	23.75±2.25[b]
14	34.38±1.06	14.88±2.42[a]	20.88±2.03[b]
21	34.88±0.99	12.38±1.41[a]	20.75±1.49[b]

Eight eyes per time point were selected in each group. Six fields of vision from each section were used for statistical analysis, and data were expressed as mean ± SD. [a]$P < 0.05$, *vs.* blank control group, [b]$P < 0.05$, *vs.* model control group (one-way analysis of variance).

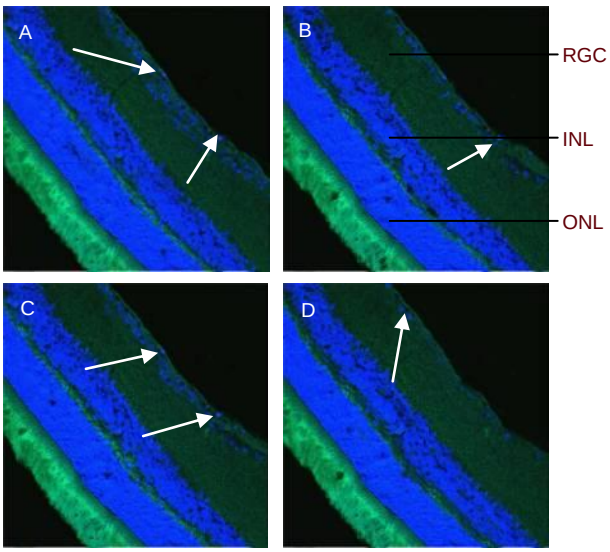

Figure 2 Apoptosis of rabbit retinal ganglion cells at 3 and 14 days after optic nerve injury (TUNEL staining, DeadEnd™ Fluorometric TUNEL System, VECTASHIELD® + DAPI, fluorescence microscope, × 1000).

After optic nerve injury, bright blue TUNEL-positive cells (arrows) appear in the retinal cell layer. Compared to corresponding time points in the model control group, the numbers of TUNEL-positive cells were reduced in the treatment group.

(A) Model control group at 3 days; (B) treatment group at 3 days; (C) model control group at 14 days; (D) treatment group at 14 days.

RGCs: Retinal ganglion cells; INL: inner nuclear layer; ONL: outer nuclear layer.

Glutamate, which is a major excitatory neurotransmitter in retina, increases rapidly following acute optic nerve injury[12]. Elevated levels of intracellular glutamate lead to programmed death of RGCs by overstimulation of NMDA and AMPA receptors, eventually resulting in a significant decrease in RGC number[14]. During development of the visual system, glutamate is involved in neural plasticity[16]. Subcutaneous injection of glutamate can destroy the retinal cell layer and inner nuclear layer[17-19]. Therefore, inhibition of glutamate neurotoxicity could potentially provide therapeutic value for acute optic nerve injury.

AMPA receptors can mediate rapid excitatory synaptic transmission, which greatly impacts neuronal integrity and synaptic plasticity. AMPA receptor activation can also regulate NMDA receptor activation[20-21]. Following optic nerve injury, AMPA receptor components altered followed by a rapid influx of calcium ions and increase in intracellular calcium ion concentration, resulting in neuronal apoptosis[22-23].

Glutamate receptor antagonists can therefore potentially reverse the excitatory toxicity of glutamate. Systematic application of glutamate receptor antagonists for the NMDA receptor and AMPA receptor could effectively treat tissue injury caused by high concentrations of glutamate after optic nerve injury, and reduce apoptosis of RGCs[24-25]. Nevertheless, there have been no reports describing use of AMPA receptor antagonists to treat acute optic nerve injury. Wang *et al* [13] reported that the AMPA/kainate (KA) receptor of adult neurons is not permeable to calcium ions, but upregulated expression of AMPA/KA or KA receptors was detected in injured RGCs[26].

The results of the present study showed that at an early stage of treatment, there were significantly more surviving RGCs in the treatment group than in the model control group. Furthermore, at each time point after injury, there were significantly fewer apoptotic cells in the treatment group than in the model control group. This suggests that at an early stage of acute optic nerve injury, AMPA receptor antagonist GYKI 52466 interacts with glutamate AMPA receptor to inhibit accumulation and inflow of calcium ion, reduce the release of related enzymes, inhibit apoptosis of RGCs[27-28], and exert effects during the later programmed apoptosis of RGCs. This could possibly occur because the AMPA receptor antagonist reduces early apoptosis of RGCs in the injury site and then reduces secondary apoptosis of RGCs in the periphery.

Taken together, our results demonstrate that following acute optic nerve injury, apoptosis of retinal ganglion cells is a programmed process that can be inhibited by an AMPA receptor antagonist.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment

Time and setting

This study was performed at The Laboratory Animal Center of the Second Affiliated Hospital of Xinjiang Medical University in China between January 2011 and November 2011.

Materials

Eighty male New Zealand rabbits, 4–5 months of age and weighing 2.0–2.5 kg, were provided by the Laboratory Animal Center, Xinjiang Medical University in

China (license No. 16-003). All rabbits were raised in a 12-hour light-dark cycle. No eye disorders were detected. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[29].

Methods

Model of traumatic optic nerve injury

Traumatic optic nerve injury was induced in the right eyes of rabbits using the following protocol. Following anesthesia by intraperitoneal injection of 3% sodium pentobarbital, rabbits were placed in a lateral position with four limbs fixed and head towards one side. Physiological saline (250 mL) supplemented with 8×10^5 U penicillin was intravenously transfused. The scalp skin was dissected using a 3–4 cm incision made on a line between tragus and lateral canthus. The temporal muscle was then bluntly dissected to expose the periosteum. After pulling away the temporal muscle using a retractor, the periosteum was dissected. The cranial bone was stripped and a bone window (2–3 cm × 2–3 cm) was made. Using a microscope, dura mater was dissected up to the bone margin, then cerebrospinal fluid was extracted. Brain tissue was slowly lifted until the white optic nerve entering into brain tissue *via* the optic foramen was exposed. The optic nerve at an approximate length of 3 mm was dissociated and occluded for five seconds using a non-traumatic vascular clamp. The resulting nerve was compressed but not fragmented. After washing with physiological saline, the skull was closed if no hemorrhage was detected. All rabbits survived during this traumatic optic nerve injury induction. After surgery, pupillary size was determined and the relative afferent pupillary defect was detected. After nerve injury induction, the animals were fed chow containing cephalexin, and erythromycin ophthalmic ointment (Beijing Shuangji Pharmaceutical Co., Ltd., China) was applied to the right eye to prevent infection.

Intervention and specimen preparation

After traumatic optic nerve injury induction, rabbits from the treatment group were intraperitoneally administered 15 mg/kg GYKI 52466 (Sigma, St. Louis, MO, USA), once a day. The control group rabbits received equal amounts of physiological saline. At 1, 3, 7, 14, and 21 days after injury induction, rabbits were sacrificed using 3% sodium pentobarbital, and eyeballs were excised and marked. After fixation for 72 hours with 4% paraformaldehyde, and dehydration and paraffin embedding, the retina was cut into 7 μm sections along the meridian of eyeballs, and the sections were stained with hematoxylin-eosin and TUNEL.

Hematoxylin-eosin staining for RGC morphology and quantitation

The sections were deparaffinized, cleared, dehydrated in a gradient ethanol series, stained with hematoxylin for 5 minutes, washed with tap water, treated in hydrogen ethanol for several seconds, washed with tap water for 1 minute, stained with eosin for 5 minutes, dehydrated, cleared, and mounted with neutral gum. Six tissues were randomly selected from each specimen. Using an optical microscope (Olympus, Tokyo, Japan) at 400 × magnification, RGCs in six areas (each area, 25 μm × 25 μm), 300 μm above and below the optic papilla were counted, and the average number of RGCs across six areas was calculated. All cell counting was done in a "single blind" manner, by a person who did not know the identity of the samples.

TUNEL detection of RGC apoptosis

The DeadEnd™ Fluorometric TUNEL System (Promega Biotechnology Co., Ltd., Beijing, China) was used for detection of apoptosis. Following deparaffinization, sections were incubated with 100 μL of 20 μg/mL proteinase K for 8–10 minutes at room temperature, washed, post-fixed with 4% formaldehyde, treated with 100 μL balanced buffer solution (200 nM postassium cacodylate, 25 nM Tris-HCl, 0.2 nM dithiothreitol, 0.25 mg/mL bovine serum albumin, 2.5 nM cobalt chloride) for 5–10 minutes, incubated with 50 μL rTdT buffer solution supplemented with 90 μL balanced buffer solution, 10 μL nucleotide-nucleoside mixture and 2 μL rTdT enzyme on ice, and then at 37°C for 60 minutes after slide placement. In the dark, sections were treated with 20 × standard saline citrate (1:10 dilution with deionized water) for 15 minutes at room temperature, washed with phosphate buffered saline, counterstained with VECTASHIELD® Mounting Medium with DAPI (Aendi Biological Science and Technology Co., Ltd., Nanjing, China), and finally observed under a fluorescence microscope.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software, and the data were expressed as mean ± SD. One-way analysis of variance was performed for comparison between groups. A value of $P < 0.05$ was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: The experimental procedure received approval by the Animal Ethics Committee, First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang Autonomous Region, China.

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