

# $\alpha$ -Aminoadipic acid protects against retinal disruption through attenuating Müller cell gliosis in a rat model of acute ocular hypertension

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**Objective:** Ocular hypertension is an important risk factor for glaucoma. The purpose of this study was to investigate the gliotoxic effects of  $\alpha$ -aminoadipic acid (AAA) in a rat model of AOH and its underlying mechanisms.

**Materials and methods:** In the rat model of acute ocular hypertension (AOH), intraocular pressure was increased to 110 mmHg for 60 minutes. Animals were divided into four groups: sham operation (Ctrl), AOH, AOH + phosphate-buffered saline (PBS), and AOH + AAA. Cell apoptosis in the ganglion cell layer was detected with the terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) assay, and retinal ganglion cells (RGCs) immunostained with Thy-1 were counted. Müller cell activation was detected using immunostaining with glutamine synthetase and glial fibrillary acidic protein. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was examined using Western blot.

**Results:** In the rat model of AOH, cell apoptosis was induced in the ganglion cell layer and the number of RGCs was decreased. Müller cell gliosis in the retinas of rats was induced, and retinal protein levels of TNF- $\alpha$  were increased. Intravitreal treatment of AAA versus PBS control attenuated these retinal abnormalities to show protective effects in the rat model of AOH.

**Conclusion:** In the retinas of the rat model of AOH, AAA treatment attenuated retinal apoptosis in the ganglion cell layer and preserved the number of RGCs, likely through the attenuation of Müller cell gliosis and suppression of TNF- $\alpha$  induction. Our observations suggest that AAA might be a potential therapeutic target in glaucoma.

**Keywords:** glaucoma, acute ocular hypertension,  $\alpha$ -aminoadipic acid, retina, Müller cells, retinal ganglion cells, TNF- $\alpha$

## Introduction

Glaucoma, a neurodegenerative disease, is characterized by gradual loss of retinal ganglion cells (RGCs) and optic nerve atrophy.<sup>1</sup> Multiple factors are related to glaucoma, such as high intraocular pressure (IOP)<sup>2</sup> and low cerebral spinal fluid pressure.<sup>3</sup> Müller cells are the major type of glial cells in the mammalian retina that can support and nourish retinal neurons, maintain extracellular ion homeostasis, glutamate recycling, and interaction in synaptic transmission.<sup>4,5</sup> Therefore, Müller cells are involved in retinal function. Investigations indicate that Müller cells not only play an important physiological role, but they are also involved in multiple pathological retinal diseases such as glaucoma.<sup>6–8</sup> It has been reported that reactive Müller cells can aggravate retinal damage by releasing cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>9</sup> Growing evidence supports that increased glial production of TNF- $\alpha$  contributes to the neurodegeneration in glaucoma.<sup>10</sup> TNF- $\alpha$  is a secreted inflammatory cytokine that

is responsible for apoptosis, necrosis, and inflammation.<sup>11,12</sup> TNF- $\alpha$  is increased in the retina following ischemia or damage and other neurodegenerative disorders.<sup>13–17</sup>

$\alpha$ -Aminoadipic acid (AAA), a six-carbon homolog of glutamate, is a well-known compound that induces specific glial toxicity through blocking the glutamate uptake.<sup>18–20</sup> The use of AAA to interfere with glial influence on neuronal tissues is reported.<sup>21,22</sup> However, the effects of AAA inhibition on Müller cell gliosis in glaucoma are unknown. The current study aimed to determine if AAA treatment inhibits Müller cell gliosis and protects against retinal abnormalities induced in a rat model of acute ocular hypertension (AOH) mimicking glaucoma. Müller cell activation of RGCs and TNF- $\alpha$  induction were examined.

## Materials and methods

### Animals

Adult male Sprague Dawley rats (weight range, 200–250 g, 8–10 weeks) were used in this study in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and followed the People's Republic of China animal care guidelines. This study was monitored and approved by the Animal Care Committee of the Capital Medical University, SCXK (Jing) 2012-0001. The animals were housed with a 12-hour light/12-hour dark cycle with standard chow and water. All surgical procedures were performed under anesthesia with intraperitoneal chloral hydrate (450 mg/kg) and topical 0.5% proparacaine hydrochloride eye drops (Alcon, Inc., Hünenberg, Switzerland).

### Rat model of AOH

The IOP was increased to 110 mmHg (14.63 kPa) for 60 minutes by using an elevated 500 mL plastic container of sterile physiological saline connected to a 27-gauge needle placed in the anterior chamber of the eye. Sham procedure eyes were treated similarly but without the elevation of the bottle; hence, the normal IOP was maintained. Time-course examination was performed at 1 day, 3 days, and 5 days after acute IOP elevation. Animals were divided into sham operation (Ctrl) group, AOH group, AOH + phosphate-buffered saline (PBS) control group, and AOH + AAA-treated group. AAA (250  $\mu$ g) was injected into vitreous humor in 5  $\mu$ L at 50 g/L concentration after 3 hours of acute IOP elevation according to the literature.<sup>23,24</sup>

### Immunohistochemistry

Tissues were fixed with 4% paraformaldehyde. Eyeballs were cryoprotected in 30% sucrose overnight at 4°C and then

embedded in optimal cutting temperature compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan). Sections (14  $\mu$ m) were incubated in 3% bovine serum albumin and 0.3% Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA) to block nonspecific binding and then incubated with primary mouse monoclonal antibodies against Thy-1 (1:200, RGC marker; Abcam, Cambridge, UK), primary mouse monoclonal antibodies against glutamine synthetase (GS; 1:100, Müller glial cell marker; Abcam), rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP; 1:200, gliosis marker; Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C, followed by incubation with secondary antibodies for 1 hour at room temperature. Nuclei were stained by Hoechst 33342. Sections were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Negative controls were incubated without primary antibodies. Slides were visualized using a confocal microscope (Leica Microsystems, Wetzlar, Germany). The intensity of immunoreactivity from photographs was analyzed using Image-Pro<sup>®</sup>Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA). Optical densities obtained from immunohistochemistry images were corrected by subtracting the average value of background noise from five image inputs.

### Terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling assay

The terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL) assay was conducted according to the manufacturer's protocol. Briefly, tissue sections were fixed in paraformaldehyde and were subsequently incubated in a TUNEL reaction medium. The immune-labeled RGCs were costained with Hoechst 33342 for 5 minutes. The sections were then mounted on microscope slides and examined under ultraviolet light using an epifluorescence microscope. Ten visual fields were analyzed per retina; the experiment was replicated three times.

### Western blot

Protein extracts were generated from retinas. Retinas from sham operation group (Ctrl), AOH group, AOH + PBS control group, and AOH + AAA group were homogenized in lysis buffer and sonicated to dissolve the tissue completely. In all, 40  $\mu$ g of total proteins from each sample were loaded per lane for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany)

and the blocked nitrocellulose membrane was incubated with a primary rabbit polyclonal antibody against TNF- $\alpha$  (1:1,000; Abcam) for 3 hours (room temperature). After washing in Tris Buffered Saline, with Tween-20 (TBST), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG prior to detection of the labeled proteins by using ECL-plus kit (PerkinElmer Inc., Waltham, MA, USA) followed by exposure of blots to X-Omat (Kodak, Rochester, NY, USA) imaging film. The images were quantified using ImageJ software (National Institutes of Health, Maryland, USA). The Gel Doc-2000 imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to perform the quantitative analysis of Western blot results. The ratio of TNF- $\alpha$  was determined by normalizing with the levels of endogenous  $\beta$ -actin. The  $\beta$ -actin band density was expressed as 100%, and the other group was expressed as percentage of that from the control group.

## Statistical analysis

The normality of the data was tested using the Shapiro–Wilk method. Analysis of variance was used to determine the difference between TNF- $\alpha$  and fluorescence intensity among three or more independent (unrelated) groups, followed by multiple comparisons using the Student–Newman–Keuls test. The Kruskal–Wallis test was used to determine the difference between TUNEL-positive cells and Thy-1-positive RGCs among three or more independent (unrelated) groups, followed by multiple comparisons using the Nemenyi test. Statistical analysis was performed using SAS 9.2 (SAS

Institute Inc., Cary, NC, USA). Values were reported as mean  $\pm$  SD. A value of  $P < 0.05$  was considered as statistically significant.

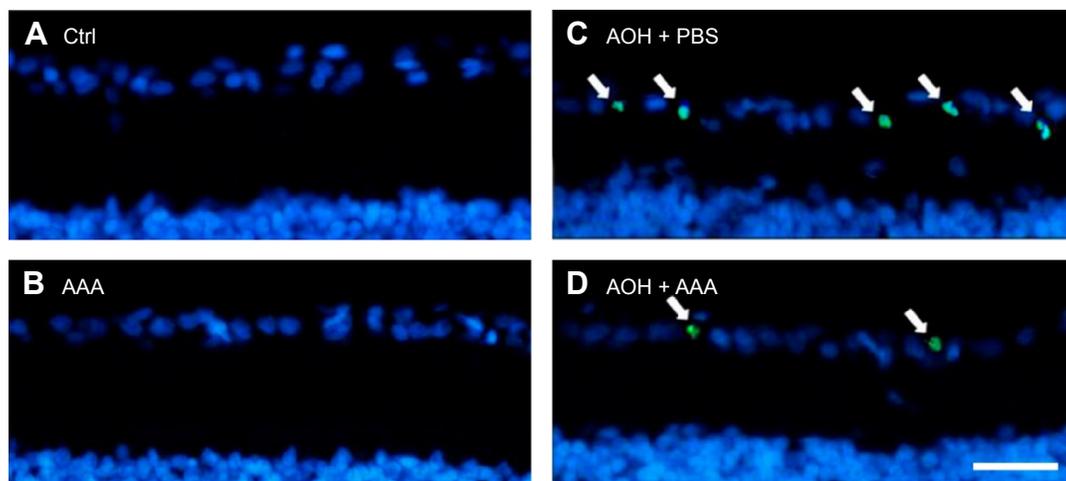
## Results

### AAA attenuates AOH-induced cell apoptosis in RGCs

To determine the effects of AAA on RGCs in the AOH model, AOH was induced in SD rats and rats were intravitreally treated with PBS, AAA, AOH + PBS, and AOH + AAA. Significantly reduced retinal thickness in the inner plexiform layer (IPL) and inner nuclear layer was observed after AOH induction versus control (Figure S1). TUNEL assay was used to examine the cell apoptosis in the retinal sections. The mean values of TUNEL-positive cells per visual field were  $0.1 \pm 0.3$  in Control (Figure 1A),  $0.2 \pm 0.4$  in AAA (Figure 1B),  $4.6 \pm 0.8$  in AOH + PBS, and  $1.7 \pm 0.7$  in AOH + AAA. AOH induced the apoptotic death of cells in the ganglion cell layer (GCL; Figure 1C). In the AOH model, intravitreal treatment with AAA attenuated the TUNEL-positive RGCs more significantly than the intravitreal treatment with PBS ( $P < 0.05$ ; Figure 1D), indicating that AAA treatment rescued RGCs from the cell apoptosis induced in the AOH model.

### AAA preserves the AOH-reduced number of RGCs

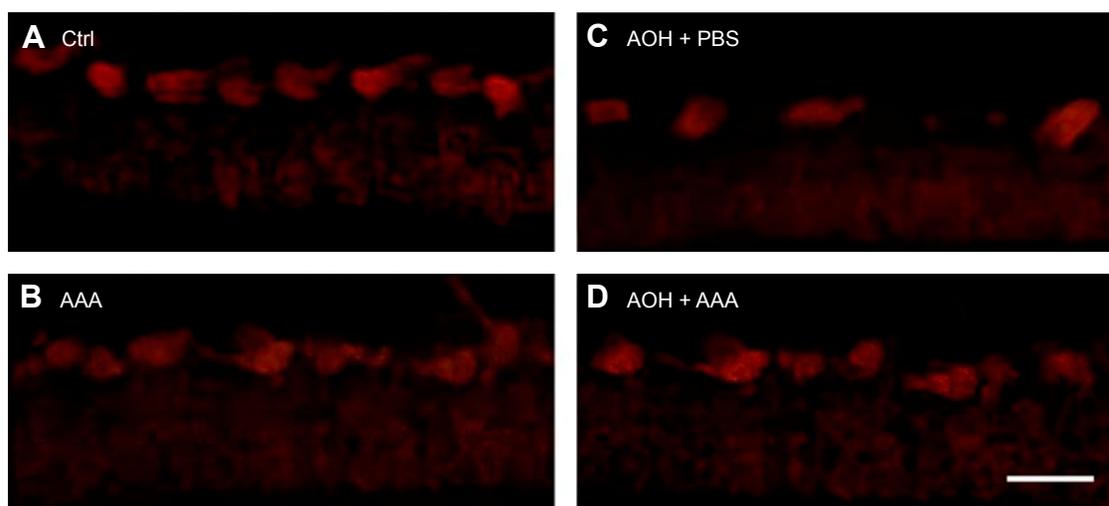
In addition, retinal sections were immunostained with antibody against Thy-1, a marker for RGCs.<sup>25</sup> The mean values of Thy-1-positive RGCs per visual field in Ctrl, AAA,



**Figure 1** AAA attenuates AOH-induced retinal apoptosis.

**Notes:** TUNEL (A–D) stainings were performed after 5 days of AOH. Cell nuclei were counterstained with Hoechst. The results showed that AOH caused significant increase in TUNEL-positive cells (C and I), but AAA could abolish this effect (scale bar = 50  $\mu$ m;  $n = 4$  per group). The green dot indicated by the arrow is an apoptotic cell and the blue areas indicate cell nuclei.

**Abbreviations:** AAA,  $\alpha$ -amino adipic acid; AOH, acute ocular hypertension; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling; Ctrl, control.



**Figure 2** AAA rescued AOH-reduced number of RGCs.

**Notes:** Retinas were collected after 5 days of AOH, and Thy-1 staining was performed. Cell nuclei were counterstained with Hoechst. The number of Thy-1-positive RGCs was quantified. In the rat model of AOH, the number of RGCs was significantly decreased and AAA restored the number of RGCs (scale bar =50  $\mu$ m; n=4 per group).

**Abbreviations:** AAA,  $\alpha$ -aminoadipic acid; AOH, acute ocular hypertension; RGCs, retinal ganglion cells; Ctrl, control; PBS, phosphate-buffered saline.

AOH + PBS, and AOH + AAA groups were  $8.2 \pm 0.9$ ,  $8.1 \pm 0.8$ ,  $3.9 \pm 0.7$ , and  $6 \pm 0.6$ , respectively. AAA treatment did not change the number of RGCs in normal conditions (Figure 2A and B). When compared with the Ctrl group (Figure 2A), the number of Thy-1-positive RGCs was decreased in the AOH + PBS group (Figure 2C), and AAA treatment significantly rescued Thy-1-positive RGCs in AOH ( $P < 0.05$ ; Figure 2D), corresponding to AAA-attenuated apoptosis of RGCs in AOH (AOH + PBS versus AOH + AAA,  $P < 0.05$ ; Figure 1).

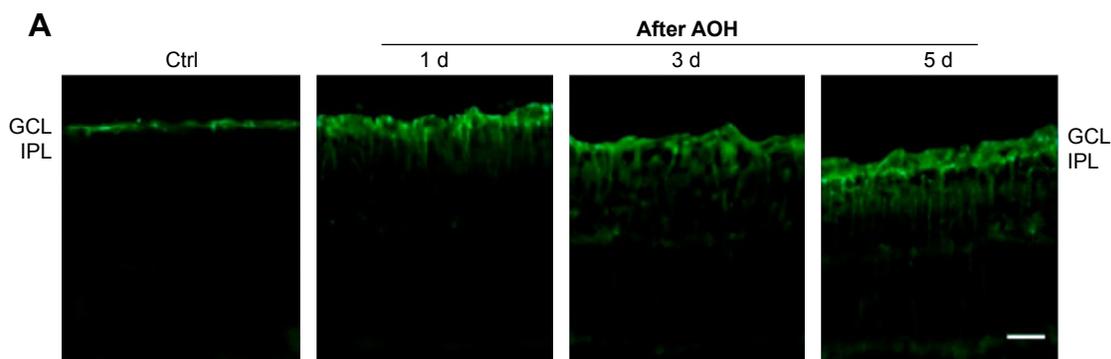
### AAA attenuates AOH-induced Müller cell gliosis

In the rat model of AOH, AAA rescued RGCs. To determine if AAA protects against AOH-induced RGC death through modulating Müller cell gliosis, immunohistochemistry for GFAP (a marker for activated glial cells) and GS (Müller cell-specific marker) was conducted.<sup>26</sup> Müller cells cover the whole retina from the nerve fiber layer to the photoreceptor

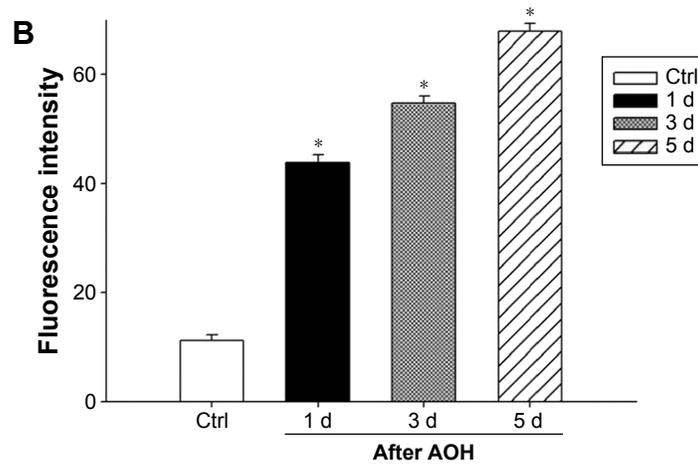
layer,<sup>27</sup> and astrocytes mainly locate along GCL. GFAP was mainly expressed along nerve fiber layer and GCL and was less or absent at other layers in the control group (Figure S2). In the rat model of AOH, colocalization of GFAP and GS was observed across IPL (Figure S2), indicating Müller cell activation. After 1 day, 3 days, and 5 days of AOH induction in rat, GFAP expression was also observed across IPL (Figure S3), indicating Müller cell gliosis. AAA treatment versus PBS control markedly reduced GFAP immunoreactivity across IPL (Figure 3), indicating attenuation in Müller cell gliosis.

### AAA decreases AOH-induced TNF- $\alpha$ production

As mentioned earlier, AAA rescued RGCs likely through the inhibition of Müller cells gliosis in the rat model with AOH. Activation of Müller cells may induce TNF- $\alpha$  production, leading to neurodegeneration in glaucoma.<sup>10</sup> To determine if AAA modulates TNF- $\alpha$  production, Western blot was



**Figure 3** (Continued)



**Figure 3** AAA attenuated induced Müller gliosis in the rat model of AOH.

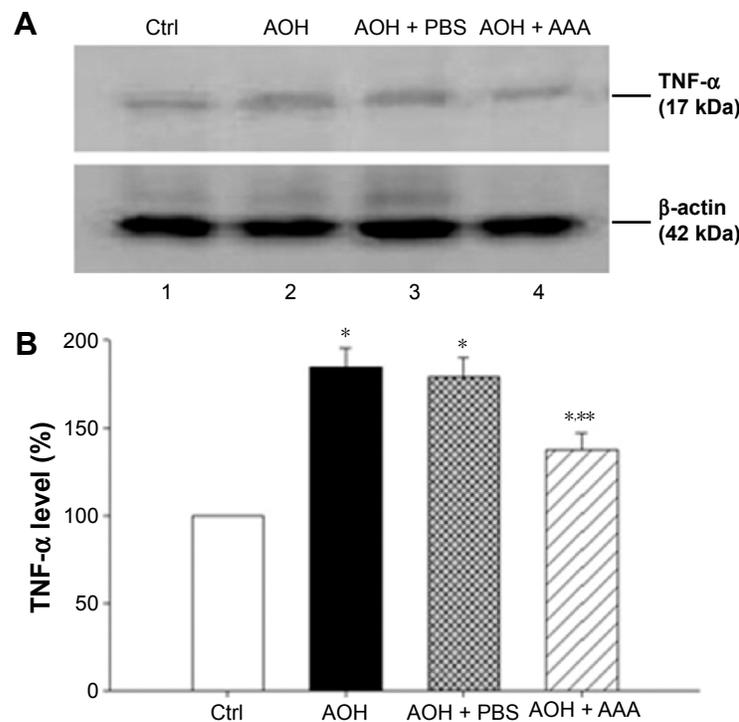
**Notes:** (A) AAA attenuated increased GFAP immunoreactivity in the Müller cells of retina of the rat model of AOH after 1 d, 3 d, and 5 d of intravitreal AAA treatment (250  $\mu$ g/eye; scale bar =50  $\mu$ m). (B) Quantitative analysis of fluorescence intensity of GFAP. \* $P$ <0.05 compared with the AOH 1 d group (n=4 per group).

**Abbreviations:** AAA,  $\alpha$ -amino adipic acid; AOH, acute ocular hypertension; GFAP, glial fibrillary acidic protein; d, day; Ctrl, control; GCL, ganglion cell layer; IPL, inner plexiform layer.

performed for the investigation of retinal protein levels of TNF- $\alpha$ . Significantly upregulated protein levels of retinal TNF- $\alpha$  were observed in AOH versus sham operation (Ctrl) group ( $P$ <0.05; Figure 4). Importantly, AAA treatment versus PBS control significantly attenuated TNF- $\alpha$  levels in the retinas of the rat model of AOH ( $P$ <0.05; Figure 4).

## Discussion

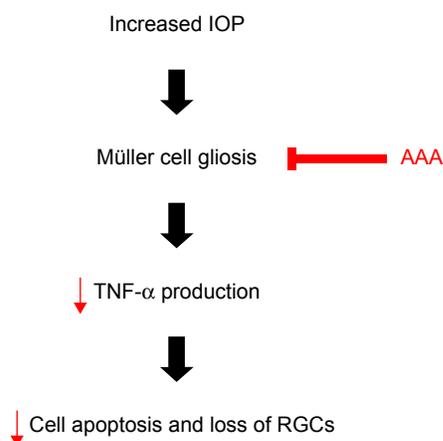
Glaucoma is a leading cause of irreversible vision loss characterized by progressive death of RGCs, and elevated IOP is a major risk factor.<sup>28</sup> A rodent model of AOH is well established for acute angle closure glaucoma, and it has been widely used to investigate the pathogenesis of death of RGCs.<sup>29</sup> In the



**Figure 4** AAA treatment reduced the induction of TNF- $\alpha$  in AOH retinas.

**Notes:** (A) Western blot results showed the effect of AAA on TNF- $\alpha$  protein expression in retinas after 5 d of AOH. (B) Quantitative analysis of protein expression levels of TNF- $\alpha$  (n=4 per group). \* $P$ <0.05 compared with the Ctrl group (no AOH induction). \*\* $P$ <0.05 compared with the AOH + PBS group.

**Abbreviations:** AAA,  $\alpha$ -amino adipic acid; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; d, days; AOH, acute ocular hypertension; Ctrl, control; PBS, phosphate-buffered saline.



**Figure 5** A schematic flow chart of AAA protection in the rat model of AOH.  
**Notes:** In the rat model of AOH, acute elevation of IOP induced Müller cell gliosis and TNF- $\alpha$  production, leading to RGC apoptosis. AAA treatment reduced Müller cell gliosis and TNF- $\alpha$  production to protect RGCs.  
**Abbreviations:** AAA,  $\alpha$ -aminoadipic acid; AOH, acute ocular hypertension; IOP, intraocular pressure; RGC, retinal ganglion cells, TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

current rat model of AOH, we found induced cell apoptosis and decreased number of RGCs with IOP induction versus sham operation control. Importantly, treatment of AAA versus PBS control significantly attenuated RGC apoptosis and rescued the reduced number of RGCs, demonstrating protective effects on AOH retinas (Figure 5).

Activated Müller cells contribute to the progression of glaucoma.<sup>30</sup> Müller cells perform a multitude of important regulatory and supportive roles, including secretion of trophic factors, removal of metabolic waste, and neurotransmitter recycling.<sup>31,32</sup> We found that Müller cells were activated with induced GFAP immunoreactivity in the rat model of AOH versus control retinas. GFAP, an intermediate filament protein, is considered as a marker of reactive Müller cell gliosis,<sup>27</sup> which is not or less expressed in Müller cells in normal retinas and expressed highly at ischemic,<sup>33</sup> light-induced retinal degeneration,<sup>34</sup> and retinal detachment.<sup>35</sup> Activation of Müller cells so far was demonstrated to have both protective and detrimental effects.<sup>36–38</sup> Especially early after injury, Müller cell gliosis is believed to be neuroprotective and promotes the repair of neurons in response to injury,<sup>36</sup> with GFAP upregulation to provide additional structural integrity to the retina at the site of injury.<sup>39</sup> However, activated Müller cells also express TNF- $\alpha$ ,<sup>40</sup> monocyte chemoattractant protein,<sup>41</sup> and nitric oxide<sup>42</sup> to induce death of RGCs. TNF- $\alpha$  induction contributes to inflammation, apoptosis, and necrosis, leading to cell death. We observed that AAA treatment attenuated GFAP immunoreactivity in Müller cell processes across IPL and reduced retinal TNF- $\alpha$  levels in the rat model of AOH. These findings suggested that AAA

promoted survival of RGCs in the rat model of AOH, likely through the inhibition of AOH-induced Müller cell gliosis and in turn downregulation of TNF- $\alpha$  protein production.

## Conclusion

We found that AAA could effectively protect retina against loss of RGCs and apoptosis in AOH retinas through attenuating Müller cell gliosis and downregulating TNF- $\alpha$  production. These observations suggested that AAA might be a potential therapeutic target in the treatment of neurodegeneration in glaucoma.

## Acknowledgments

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## Disclosure

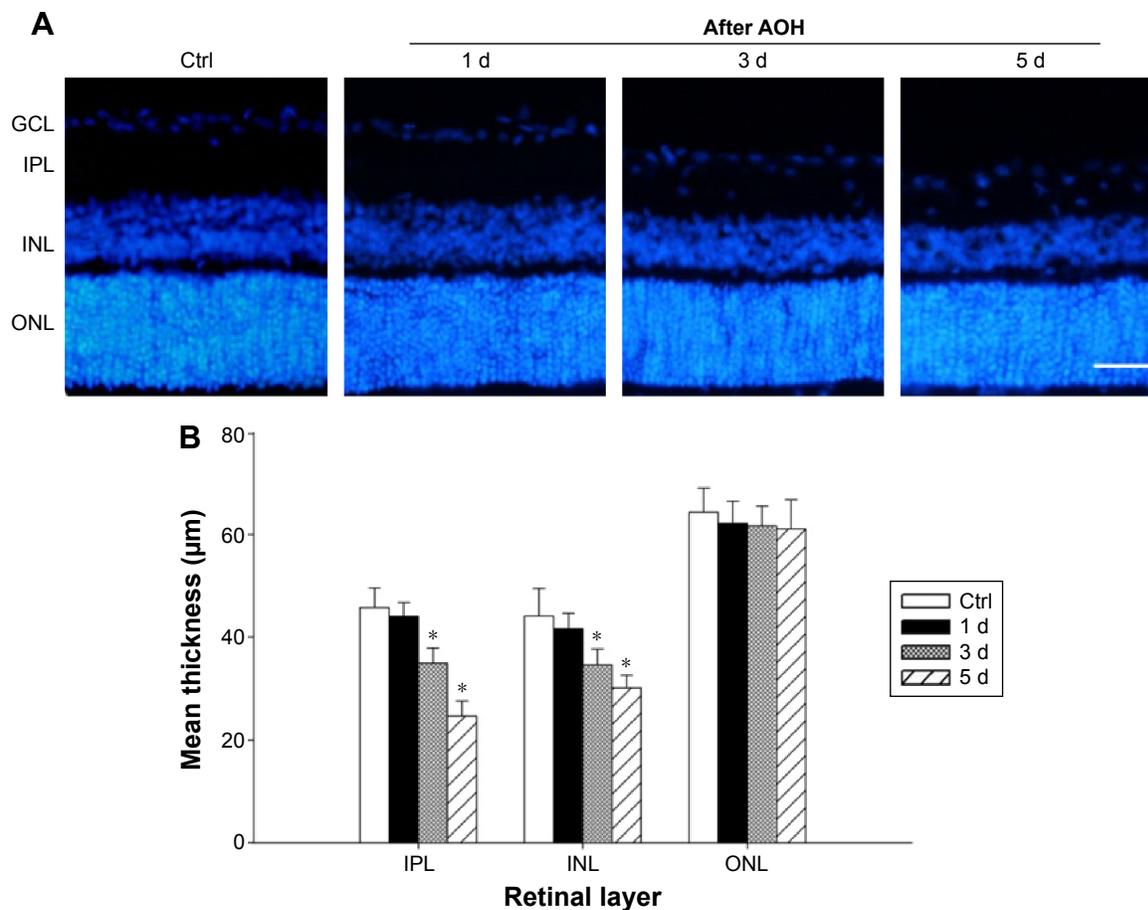
The authors report no conflicts of interest in this work.

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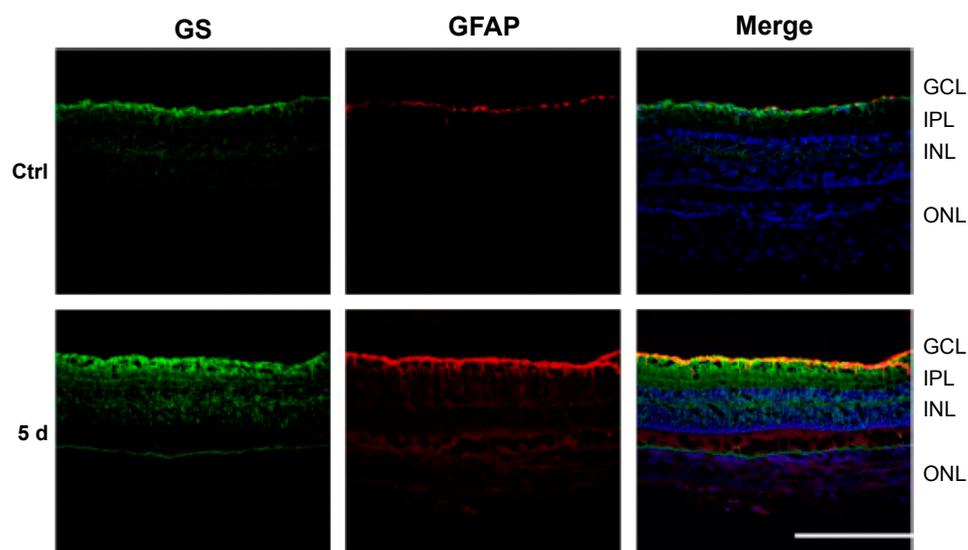
## Supplementary materials



**Figure S1** (A) Representative results of Hoechst staining in rat retina after 1 d, 3 d, and 5 d of AOH; (B) Quantitative analysis of the retinal thickness.

**Notes:** The results showed that the thickness of IPL and INL layers decreased significantly following the reperfusion time after AOH treatment (scale bar =50  $\mu\text{m}$ ). \* $P < 0.05$  compared with the control group (n=4 per group).

**Abbreviations:** AOH, acute ocular hypertension; IPL, inner plexiform layer; INL, inner nuclear layer; Ctrl, control; d, day; GCL, ganglion cell layer; ONL, outer nuclear layer.



**Figure S2** Immunohistochemistry costained with antibodies against GS (green) and GFAP (red).

**Note:** Strong expression of GFAP was shown in the GS-positive Müller cell after 5 d of AOH compared with the control group (scale bar =50  $\mu\text{m}$ ).

**Abbreviations:** GS, glutamine synthetase; GFAP, glial fibrillary acidic protein; AOH, acute ocular hypertension; Ctrl, control; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; d, days.

