Regulatory effects of inhibiting the activation of glial cells on retinal synaptic plasticity

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Abstract
Various retinal injuries induced by ocular hypertension have been shown to induce plastic changes in retinal synapses, but the potential regulatory mechanism of synaptic plasticity after retinal injury was still unclear. A rat model of acute ocular hypertension was established by injecting saline intravitreally for an hour, and elevating the intraocular pressure to 14.63 kPa (110 mmHg). Western blot assay and immunofluorescence results showed that synaptophysin expression had a distinct spatiotemporal change that increased in the inner plexiform layer within 1 day and spread across the outer plexiform layer after 3 days. Glial fibrillary acidic protein expression in retinae was greatly increased after 3 days, and reached a peak at 7 days, which was also consistent with the peak time of synaptophysin expression in the outer plexiform layer following the increased intraocular pressure. Fluorocitrate, a glial metabolic inhibitor, was intravitreally injected to inhibit glial cell activation following high intraocular pressure. This significantly inhibited the enhanced glial fibrillary acidic protein expression induced by high intraocular pressure injury. Synaptophysin expression also decreased in the inner plexiform layer within a day and the widened distribution in the outer plexiform layer had disappeared by 3 days. The results suggested that retinal glial cell activation might play an important role in the process of retinal synaptic plasticity induced by acute high intraocular pressure through affecting the expression and distribution of synaptic functional proteins, such as synaptophysin.

Key Words: nerve regeneration; neuronal plasticity; retina; synapses; synaptophysin; glial cells; high intraocular pressure; fluorocitrate; glial fibrillary acidic protein; NSFC grant; neural regeneration

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Introduction
Different types of retinal damage [1-8], including acute and chronic ocular hypertension, optic nerve transection, retinitis pigmentosa, age-related macular degeneration and retinal detachment could induce plastic changes of retinal synapses, which might precede the serious damage of neuron somas [9-10]. Our previous studies also found that besides the death of ganglion cells, acute high intraocular pressure could induce plastic changes of retinal synapses: synapsyn elements showed a distinct spatiotemporal pattern of changes from the internal to external retinal layers [4, 11]. These morphological and functional changes to synapses after retinal injury could explain why many intervention measures protected neurons from death but failed to fully recover the damaged visual function [12-15]. Therefore, it is necessary to investigate both the protection of synapses as well as protecting neurons from death. Accumulated evidences indicated that spontaneous retinal waves [16-17], visual experience [18-19], light stimulation [20] and neuronal trophic factors [21-22] all play important roles in the developmental plasticity of the mammalian retina. It has also been reported that appropriate neuronal Nogo-66 receptor 1 antagonism may reduce retinal ganglion cells loss and attenuate synaptic degeneration in chronic glaucoma [23]. The Rho kinase inhibitor significantly blocked rod axonal retraction after retinal detachment [24]. Brain-derived neurotrophic factor may control dopamine release and influence the network of dopaminergic amacrine cells, thereby balancing the rod and cone pathway in rat retina after axotomy of optic nerve transection [25]. However, to date a key question still remains unanswered: What are the potential regulatory mechanisms triggering synaptic plasticity after retinal injury? This needs to be resolved to enable research progress in retinal injury and repair.

The traditional view is that the functions of glial cells in central nervous system are primarily passive and provide substrates for energy metabolism and support the physiology of associated neurons by regulating ion concentrations and taking up neurotransmitters [26]. It is becoming increasingly clear that glial cells can no longer be regarded as passive bystanders at the synapse [27]. Glial cells, especial-
ly astrocytes, actively communicate with presynaptic and postsynaptic neurons and influence synaptic morphology, number and function which have long been thought to be under the control of neurons exclusively. Astrocytes are often found in close apposition to the presynaptic and postsynaptic machinery of neurons at the excitatory (glutamatergic) connections. The intimate vicinity of synapses and astrocytes inspired the concept of "tripartite synapses", which regarded glial cells as integral elements of synaptic connections. Glial cells are found to be involved in the synaptic signal transmission and integration, impacting on synapse formation and maturation and potentially regulating synaptic plasticity and network excitability. This raised a question whether glial cells play roles in the process of synaptic plasticity induced by retinal injury.

Fluorocitrate is an inhibitor of glial cell metabolism and can selectively inhibit the tricarboxylic acid cycle by blocking aconitase, a metabolic enzyme only used in glia. A relatively narrow dose range of fluorocitrate can result in a reversible impairment of reactive glial metabolic activity and caused no destruction of neurons or effect on astrocytes in normal tissues. For spinal cord injury and brain injury, fluorocitrate is commonly administered by intrathecal injection and can efficiently inhibit glial activation induced by the injury. Previously, Virgili et al. reported that fluorocitrate by intravitreal injection could lead to a temporary impairment of Müller cell metabolism in the retina.

In the present study, we took an animal model of ischemia-reperfusion injury induced by acute high intraocular pressure as an example and metabolically inhibited glial activation with fluorocitrate by intravitreal injection to investigate the possible roles of glial cells in the process of synaptic plasticity after retinal injury.

Results

Quantitative analysis of experimental animals

A total of 156 healthy adult Sprague-Dawley rats were included in this experiment. One animal died after anesthesia, two died after induction of high intraocular pressure, and three rats were deemed invalid because of additional damage to their eyes. The remaining 150 rats were randomly divided into a sham surgery group, an acute high intraocular pressure group, a vehicle-treated high intraocular pressure group and a fluorocitrate-treated high intraocular pressure group. The intraocular pressures of the left eyes of the latter three groups were gradually elevated to 110 mmHg by injection of normal saline into the anterior chamber and maintained for 60 minutes then slowly decreased to the normal level. In the sham surgery group, a needle was inserted into the anterior chamber of the eye without elevating the pressure. Five rats in each group were examined at each time point for immunofluorescence detection. At 7 days, in each of the acute high intraocular pressure and fluorocitrate-treated high intraocular pressure group, five rats were used for western blot. The unoperated right eyes served as the normal control group. A total of 150 rats were included in the final analysis.

Glial fibrillary acidic protein expression in retinas of the acute high intraocular pressure group

Immunolabeling of retinal glial cells was performed with an antibody to glial fibrillary acidic protein. In healthy adult rat retinae, glial fibrillary acidic protein was primarily confined to the somas of astrocytes in nerve fiber layer and ganglion cell layer, but not in the Müller cells. The distribution of glial fibrillary acidic protein expression did not change much within 1 day after high intraocular pressure. However, glial fibrillary acidic protein expression was greatly increased by 3 days following induction of high intraocular pressure with a distribution not only in astrocytes but also in the cell bodies and processes of Müller cells in the retina. The number of glial fibrillary acidic protein-immunoreactive processes gradually increased and appeared all over the retinal layers reaching as far as the outer nuclear layer and the outer limiting membrane, with a peak on the seventh day after high intraocular pressure was induced (Figure 1). Glial fibrillary acidic protein immunoreactivity in the sham surgery group was similar to that in the normal control group (images not shown).

Results of western blot assay showed that glial fibrillary acidic protein expression was easily detectable as a clear 50 kDa band in healthy adult rat retinae. Glial fibrillary acidic protein expression was greatly increased and the band was blacker and thicker at 7 days in the acute high intraocular pressure group compared with that in the normal control group (P < 0.01; Figure 2).

Glial fibrillary acidic protein expression in retinas of the fluorocitrate-treated high intraocular pressure group

After treatment with fluorocitrate, glial fibrillary acidic protein expression was still highly distributed in the cell bodies and processes of astrocytes in the nerve fiber layer and the ganglion cell layer of rat retina within 7 days after high intraocular pressure. No increased glial fibrillary acidic protein-immunoreactive processes were detected in Müller cells. The distributional pattern of glial fibrillary acidic protein showed no obvious change at 3 and 7 days following fluorocitrate-treated high intraocular pressure injury. At 14 days, the glial fibrillary acidic protein-immunoreactive processes were moderately increased, but did not extend into the outer retina (Figure 1). The glial fibrillary acidic protein expression at the various periods in vehicle-treated high intraocular pressure group was similar to that of the retina in the acute high intraocular pressure group (image not shown).

Western blot assay showed that, compared with the acute high intraocular pressure group, glial fibrillary acidic protein in fluorocitrate-treated high intraocular pressure group was much less at 7 days (P < 0.01). No significant difference in glial fibrillary acidic protein expression was detected between the fluorocitrate-treated high intraocular pressure group and normal control group (P > 0.05; Figure 2).

Synaptophysin expression in the retinas of the acute high intraocular pressure group

In the healthy adult rat retinae, synaptophysin immunoreactivity was prominently distributed in the outer plexiform layer and the inner plexiform layer, with a stronger staining in the outer plexiform layer and punctate staining in the inner margin of inner plexiform layer. The expression of synaptophysin after high intraocular pressure induction showed a tendency of increase from the inner plexiform layer to the outer plexiform layer. Within a day following acute high...

Intraocular pressure, synaptophysin expression was slightly increased in the inner plexiform layer and the immunofluorescence intensity of synaptophysin was higher than normal control group. Weak synaptophysin immunoreactivity was also seen in the inner nuclear layer. Synaptophysin immunoreactivity was still detectable across the entire inner plexiform layer although the retinal ganglion cells in ganglion cell layer had selectively died and the inner plexiform layer had clearly thinned following 3 days of high intraocular pressure induction. In the outer plexiform layer, the expression and distribution pattern of synaptophysin were similar to that in the normal control group within 1 day. After 3 days of high intraocular pressure induction, synaptophysin immunoreactivity had spread and extended into the inner part of the outer nuclear layer, reaching the maximum distribution in the outer plexiform layer and outer nuclear layer by 7 days (Figure 3).

Synaptophysin expression in the inner plexiform layer was determined using measurements of the relative average gray value (Figure 4). Statistical analysis showed that the value of synaptophysin at various periods of high intraocular pressure induction was lower than in the normal control group ($P < 0.05$), indicating that the fluorescence intensity of synaptophysin immunoreactivity in the inner plexiform layer steadily increased after high intraocular pressure injury. The results for the positive area percentage of synaptophysin in the outer plexiform layer and outer nuclear layer after high intraocular pressure showed no obvious change within 1 day compared with normal control, but it was significantly higher than normal control in the outer plexiform layer and outer

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**Figure 1** Fluorescence immunoreactivity of GFAP in rat retina at different survival times under a laser scanning fluorescence microscope. The immunofluorescence dye is Cy3. Nuclei of the cells were stained by Hoechst solution (blue). GFAP expression did not change much within 1 day after HIOP and was greatly increased extending into the outer retina at 3 days with a peak at 7 days. GFAP: Glial fibrillary acidic protein; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; NFL: nerve fiber layer; HIOP: high intraocular pressure; FC: fluorocitrate. 2 h, 6 h, 12 h, 1 d, 3 d, 7 d, 14 d indicate 2, 6 and 12 hours, and 1, 3, 7 and 14 days after HIOP injury, respectively; Normal: normal control group. Scale bars: 20 μm.

**Figure 2** Western blot assay for expression of GFAP protein in rat retinas at 7 days of acute HIOP group and fluorocitrate-treated HIOP group. (A) Western blot bands of GFAP protein at 7 days in rat retinas of acute HIOP group and fluorocitrate-treated HIOP group. (B) The relative expression levels of GFAP proteins in rat retina. The relative expression level of GFAP protein was normalized against β-actin and presented as the ratio of the values of the experimental group to the normal control group. Data are presented as mean ± SD. $^aP < 0.01$, vs. normal control (one-way analysis of variance). $^bP < 0.01$, vs. acute HIOP group (independent sample t-test). GFAP: Glial fibrillary acidic protein; HIOP: high intraocular pressure; FC: fluorocitrate; Normal: normal control group.
nuclear layer after 3 and 7 days, reaching the maximum at 7 days after high intraocular pressure induction \((P < 0.01; \text{Fig.} \ 4)\). Synaptophysin immunoreactivity in the sham surgery group was similar to the normal group (images not shown).

**Synaptophysin expression in the retinas of fluorocitrate-treated high intraocular pressure group**

After treatment with fluorocitrate, synaptophysin expression was confined to the outer plexiform layer and the entire inner plexiform layer (Figure 3). Compared with the high intraocular pressure group, at the time points within 1 day, synaptophysin expression was reduced in the inner plexiform layer and the intensity of immunofluorescence was clearly weakened in the fluorocitrate-treated high intraocular pressure group. The intensity of synaptophysin immunoreactivity was similar to the normal control group in the inner plexiform layer at 3 days. During this period, the inner retinal structure had become progressively thinner. In the outer plexiform layer of the rat retinas of fluorocitrate-treated high intraocular pressure group, the intensity and distribution of synaptophysin were similar to the normal control up to 1 day. However, it was worth noticing that synaptophysin immunoreactivity in the outer plexiform layer of the fluorocitrate-treated retina was still confined to the...
outer plexiform layer at 3 days. Wider distribution of synaptophysin in the outer plexiform layer and outer nuclear layer after high intraocular pressure induction was not detected in the retina after treatment with fluorocitrate (Figure 3). The synaptophysin expression at the various periods in vehicle-treated high intraocular pressure group was similar to that of the retina with high intraocular pressure (image not shown).

Statistical analysis showed that the relative average gray value of synaptophysin immunoreactivity in the inner plexiform layer was higher in the fluorocitrate-treated high intraocular pressure group than that in the high intraocular pressure group at the corresponding time points within 1 day ($P < 0.05$), indicating that the fluorescence intensity of synaptophysin immunoreactivity in the inner plexiform layer was greatly decreased after fluorocitrate-treated high intraocular pressure injury. At various periods after treatment with fluorocitrate, the positive area percentage of synaptophysin immunoreactivity in the outer nuclear layer and outer plexiform layer was similar to normal control group ($P > 0.05$; Figure 4). Compared with the corresponding high intraocular pressure group, the positive area percentage of synaptophysin in the outer plexiform layer and outer nuclear layer had significantly decreased at 3 and 7 days after treatment with fluorocitrate ($P < 0.01$).

Discussion
The ability of neurons to form synapses has long been assumed to be intrinsic to neurons. Yet, growing evidence suggests that not all plasticity events can be explained solely by neuronal elements but that glial cells provide an essential contribution [26, 38]. Glial cells have been demonstrated to play important roles in synaptic remodeling and exert powerful control over the number of synapses in the central nervous system. Glial cells around synapses are essential for presynaptic and postsynaptic functions and are required for synaptic stability and maintenance [26, 38-55]. These effects were thought to be in part through glial release of a wide variety of neuroactive gliotransmitters and diffusible factors, such as thrombospondins, D-serine, and glypicans, during physiological and pathological conditions [39-40]. It is also now widely accepted that glial cells are considered as participants in the activity-dependent structural and functional synaptic changes, modulating and even mediating synaptic scaling, homeostatic plasticity, metaplasticity, long-term potentiation and long-term depression in the development, injuries and diseases of the central nervous system [26, 46-55].

The retina is an accessible part of the central nervous system and has often served as a model system for the study of brain injury and repair. Accumulating evidence has shown that glial cells in the retina are strongly activated in response to different types of damage to the retina, including retinal detachment, chronic glaucoma and ischemia-reperfusion induced by elevated intraocular pressure [26, 60]. Increased expression of glial fibrillary acidic protein within macroglia is commonly seen as a hallmark of glial activation after damage within the central nervous system, including the retina [61]. Our present study observed that glial fibrillary acidic protein immunoreactivity was greatly increased, with a peak at 7 days after high intraocular pressure. The increased glial fibrillary acidic protein was not only expressed by astrocytes in the nerve fiber layer and ganglion cell layer, but also distributed in cell bodies and processes of Müller cells reaching as far as the outer nuclear layer. Western blot assay also showed that glial fibrillary acidic protein expression was significantly enhanced in rat retina after 7 days of high intraocular pressure-induced injury. We also noticed a steady increase with time in glial fibrillary acidic protein mRNA expression from 6 hours to 7 days after high intraocular pressure injury in rat retina in a preliminary gene-chip analysis (our own unpublished data). These data from transcript and protein levels indicated that glial cells were activated in rat retina after high intraocular pressure injury. Synaptophysin, a marker of presynaptic terminals, is widely used in the study of synaptic plasticity in the central nervous system and synaptophysin immunoreactivity has been considered to correlate well with synaptogenesis in the central nervous system [62]. Our previous studies demonstrated that high intraocular pressure-induced retinal injury could result in plastic changes of retinal synapses [26, 41-63]; synaptophysin expression had a distinct spatiotemporal pattern from the inner plexiform layer to the outer plexiform layer and ultrastructure assay detected bouton-like vesicle-containing structures in the outer nuclear layer, however, the presynaptic changes were not accompanied by changes in the postsynaptic components. In this present study, the results of synaptophysin expression in the high intraocular pressure group and the sham surgery group were consistent with our previous study that synaptophysin expression in the inner plexiform layer was initially increased after high intraocular pressure injury and then synaptophysin distribution in the outer plexiform layer spread out, reaching as far as the inner outer nuclear layer with a peak at 7 days. The aforementioned coincidence in the spatiotemporal tendency of glial activation and synaptic changes after retinal injury induced by high intraocular pressure raised a question: whether glial cells played a regulatory role in the process of synaptic plastic changes after retinal injury induced by high intraocular pressure.

The present study showed that glial cell activation induced by high intraocular pressure was strongly inhibited by intravitreal injection of fluorocitrate. There was no apparent increase in glial fibrillary acidic protein immunoreactivity after treatment with fluorocitrate, even at 7 days, when, in the high intraocular pressure group, glial fibrillary acidic protein expression reached a peak with its immunoreactive processes extending to the outer limited membrane. It suggested that fluorocitrate administrated by intravitreal injection can get rapidly and easily to the retina and effectively inhibit glial activation induced by high intraocular pressure. Intriguingly, after treatment with fluorocitrate, with the inhibition of glial activation, synaptophysin expression in rat retina was also obviously reduced in the inner plexiform layer within 1 day and the widened distribution of synaptophysin disappeared in the outer plexiform layer and outer nuclear layer at 3 and 7 days, whereas it was be easily detectable in high intraocular pressure retinas. The above results indicated that the inhibition of glial activation could significantly decrease the
up-regulation of synaptophysin expression and the broadening of synaptophysin distribution in the outer plexiform layer induced by high intraocular pressure in rat retina. We speculate that glial cell activation might influence synaptic plasticity of rat retina with high intraocular pressure injury through promoting the expression and distribution of synaptic functional proteins, such as synaptophysin. It has been reported that in blue light injured rat retina, activated retinal Müller cells could up-regulate the expression of SAP97, a member of the membrane associated guanylate kinase protein family, which is thought to play key roles in synapse assembly and synaptic plasticity [64]. Reactive astrocytosis can cause a remarkable imbalance of glutamatergic/GABAergic compartments in the neuronal network and induce perturbation of synaptic homeostasis in spared sciatic nerve injury model [65]. It remains to be explored whether the activated glial cells impact on other aspects of synapses besides presynaptic proteins in the process of synaptic plasticity after high intraocular pressure-induced retinal injury.

The present study indicated the following points. Retinal glial cells were strongly activated after high intraocular pressure-induced retinal injury, while intravitreal injection of fluorocitrate could greatly inhibit glial activation. The reduced expression of synaptophysin in the inner plexiform layer and outer plexiform layer after treatment with fluorocitrate implicated that glial cells might strongly influence synaptic plasticity through modulating the expression and distribution of synaptic related proteins, then regulating synaptic number and function. These highlighted glial cells as potential novel targets for protection of synapses after retinal injury. In the brain, astrocyte activation was involved in synaptic plasticity and functional recovery [66-67]. It may be that through secreting TSP1/2 and binding to its neuronal receptor α2δ1 astrocytes can influence synaptogenesis in the central nervous system [68]. Evidence emerged that glial cells can also promote synapse formation of cultured retinal ganglion cells through boosting the development of presynaptic elements by releasing synaptogenic factors, such as cholesterol [69]. Recent reports demonstrated that astrocytes could control the formation, maturation and plasticity of synapses through regulating the balance between hevin, which is a positive regulator of synapse formation and triggered excitatory synaptogenesis, and a secreted protein acidic and rich in cysteine, which was a negative regulator of synapse formation and antagonized synaptogenic function of hevin [70]. Earlier evidences indicated that glial cells are capable of modulating synaptic transmission and neuronal excitability by evoking Ca\(^{2+}\) waves or a sustained hyperpolarizing current within the retina and thus may contribute to information processing in the retina [71]. Future study is warranted to investigate the detailed potential possible mechanisms by which glial cells modulate retinal synaptic plasticity after retinal injury.

Materials and Methods
Design
A randomized controlled animal experiment.

Time and setting
The experiment was performed at the Laboratory of the Department of Anatomy and Neurobiology, Xiangya School of Medicine, Central South University, China between March and December 2012.

Materials
Healthy adult Sprague-Dawley rats, of equal numbers of each gender, weighing 180–220 g were obtained from the Animal Center of Agricultural University of Hunan (Permission No. SCXK (Xiang) 2009-0004). They were allowed free access to food and water. The temperature and ambient humidity were maintained at 25°C and 50–55%, respectively. The animal room was illuminated from 7:00 to 19:00. All protocols for animal use were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 1985 revision (National Institutes of Health Publication. No. 86-23).

Methods
Establishment of acute high intraocular pressure model in rats
Acute high intraocular pressure animal model was established according to a previously published procedure [72]. The rats were anesthetized by an intraperitoneal injection with a 1:1 mixture solution (0.5 mL/100 g) of 10% chloral hydrate and 25% urethane. A sterile disposable intravenous infusion needle, connected to an instillation instrument filled with normal saline, was inserted into the anterior chamber through the lateral border of the left eye. The intraocular pressure was elevated to 14.63 kPa (110 mmHg) and maintained for 60 minutes, then slowly decreased to a normal level. A drop of chloramphenicol eye drop was administered to the conjunctival sac against infection. Rats with cataracts or inflammation or ocular fundus hemorrhage in the eyes were excluded and considered as unsuccessful models. For the sham surgery group, the intraocular pressure was kept in 20 mmHg without elevation.

Fluorocitrate injection
Fluorocitrate was dissolved initially in 2 mol/L hydrochloric acid and then diluted with 10 mmol/L PBS to attain a final concentration of 16 mmol/L before administration [73]. After the induction of high intraocular pressure, the head was fixed on the three-dimensional position indicator. The disinfect microsyringe was inserted into the vitreous chamber along the lateral border of corneal and sclera, and then 2 μL of fluorocitrate or vehicle (0.01 mol/L PBS) was injected into the vitreous chamber after extracting the equivalent volume of vitreous body. The needle of microsyringe was kept in the vitreous chamber for 2 minutes and then slowly removed. The fluorocitrate or vehicle was administrated repeatedly at 4, 8 and 11 days by intravitreal injection.

Tissue preparation
At the indicated time points, rats were anesthetized and transcardially perfused with 150 mL of 0.9% normal saline followed by 350 mL of 4% formaldehyde. After perfusion, the eyeballs were dissected out, the cornea and lens were removed and the eyecups were post-fixed in the same fixative overnight at room temperature and subsequently replaced with 15% sucrose for 4 hours and then immersed in 30%...
sucrose overnight at 4°C. The eyecups were subsequently embedded in Tissue-Tek optimal cutting temperature medium. Cryosections with 14-μm thickness were cut on a cryostat (Thermo Electron Corporation, Cheshire, UK) and the sections containing the optic nerve were mounted on gelatin-coated slides and stored at −20°C until use.

For western blot analysis, the animals were sacrificed with excessive chloral hydrate (0.5 mL/100 g) intraperitoneally at 7 days following high intraocular pressure. The eyeballs were dissected out, and the cornea, lens and sclera were removed on an ice-cold plate. The retinal tissues were weighed and cryopreserved at −80°C until further use.

**Immunofluorescence for glial fibrillary acidic protein and synaptophysin expression in the retinal tissues of rats**

The slides were thawed and washed three times for 10 minutes with 0.01 mol/L PBS containing 0.1% Tween-20. Subsequently, the sections were incubated with blocking buffer containing 5% normal bovine serum and 0.3% Triton X-100 in 0.1 mol/L phosphate buffer for 1 hour at room temperature. The sections were then incubated with mouse anti-rat glial fibrillary acidic protein monoclonal antibody (1:6,000, Calbiochem, Darmstadt, Germany) or mouse anti-rat synaptophysin monoclonal antibody (1:4,000, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. After washing, the sections were incubated with Cy3-conjugated AffiniPure donkey anti-mouse IgG (1:400; Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours at room temperature. Cell nuclei were stained for 8 minutes with Hoechst 33258 staining solution (Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China). Finally, the sections were mounted in 1:1 0.1 mol/L phosphate buffer and glycerol (by volume) and stored at 4°C in the dark. Negative controls performed with the secondary antibody alone showed no staining. All images were captured using a laser scanning fluorescence microscope (Nikon 80I, Tokyo, Japan).

**Western blot assay for glial fibrillary acidic protein expression in the retinal tissues of rats**

The retinal tissues were homogenized by an ultrasound homogenizer at a ratio of 1:10 (g/mL) in lysis buffer with Tissue Protein Extraction Reagent with phenylmethylsulfonyl fluoride (PMSF, CWBIO Technology, Beijing, China). Protein quantification was performed using bicinchoninic acid protein assay kit (CWBIO Technology) in duplicate and the results were averaged. The protein samples (40 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in PBS containing 0.3% Triton X-100 for 1 hour at room temperature and incubated overnight at 4°C with a mouse anti-rat glial fibrillary acidic protein monoclonal antibody (1:6,000; Calbiochem, Darmstadt, Germany) or mouse anti-rat β-actin monoclonal antibody (1:2,000; Beyotime Institute of Biotechnology) in 5% non-fat milk. The blots were subsequently incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (1:15,000; Zhongshan Goldenbridge Biotechnology, Beijing, China), developed in high sensitivity chemiluminescence reagent (CWBIO Technology) for 5 minutes and exposed onto Kodak BioMax Hyperfilm (Kodak, Shanghai, China) for 1 to 3 minutes. Nonspecific staining was not detected when the primary antibodies were excluded. Glial fibrillary acidic protein bands and β-actin bands were scanned (BenQ 8550T, Taiwan, China).

**Image processing**

Two sections were selected from each retina. Four regions symmetric to the optic nerve (two consecutive regions on each side) were chosen from each section for image acquisition at 40 × magnification. Images of 2,560 × 1,920 pixels (width × height) containing the designated area were selected for statistical analysis. The average gray value of synaptophysin immunoreactivity in the inner plexiform layer was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA) [3-4]. The average gray value was negatively correlated with the fluorescent intensity of synaptophysin immunoreactivity. The average gray values of the experimental groups at different time points were compared with that of normal control group (100%) on the same slide. The normalized percent average gray value was defined as a relative average gray value. The positive area of synaptophysin immunoreactivity in the outer nuclear layer and outer plexiform layer was measured blindly using ImageJ software [5-6]. The positive area percentage of synaptophysin immunoreactivity relative to the total area of the outer plexiform layer and outer nuclear layer was obtained.

For western blot analysis, the integrated density value for the intensity of the glial fibrillary acidic protein-immunoreactive bands was quantified by Fluor Chem 8900 software (Alpha Innotech Corporation, San Leandro, CA, USA). The relative levels of glial fibrillary acidic proteins were normalized against β-actin and presented as the ratio of the values of the experimental group to normal control group (=1).

**Statistical analysis**

The measurement data were presented as mean ± SD. One-way analysis of variance and the independent sample t-test was used to analyze the data using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). A value of P < 0.05 was considered statistically significant. The statistical graphs were performed by GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA, USA).

**Author contributions:** Zhou LH performed the majority of the experiments, provided data, and performed data analysis, wrote the manuscript. Wang H revised the manuscript. Luo J conducted a part of the animal model. Xiong K and Zeng LP gave some good advice to the experimental design and final manuscript. Chen D designed the study, oversaw the experiment, prepared the manuscript, revised the manuscript and obtained funding. Huang JF obtained funding, revised and finally approved the manuscript. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Peer review:** Glial cells play important roles in the formation and remodeling of retinal synapses. The authors further confirmed that
the expression of synaptophysin in retina was affected when glial activation was inhibited.

References


