Expression of Glutamate and GABA during the Process of Rat Retinal Synaptic Plasticity Induced by Acute High Intraocular Pressure

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Acute high intraocular pressure (HIOP) can induce plastic changes of retinal synapses during which the expression of the presynaptic marker synaptophysin (SYN) has a distinct spatiotemporal pattern from the inner plexiform layer to the outer plexiform layer. We identified the types of neurotransmitters in the retina that participated in this process and determined the response of these neurotransmitters to HIOP induction. The model of acute HIOP was established by injecting normal saline into the anterior chamber of the rat eye. We found that the number of glutamate-positive cells increased successively from the inner part to the outer part of the retina (from the ganglion cell layer to the inner nuclear layer to the outer nuclear layer) after HIOP, which was similar to the spatiotemporal pattern of SYN expression (internally to externally) following HIOP. However, the distribution and intensity of GABA immunoreactivity in the retina did not change significantly at different survival time post injury and had no direct correlation with SYN expression. Our results suggested that the excitatory neurotransmitter glutamate might participate in the plastic process of retinal synapses following acute HIOP, but no evidence was found for the role of the inhibitory neurotransmitter GABA.

Key words: high intraocular pressure, retina, synaptophysin, glutamate, GABA

I. Introduction

Normal visual function depends on the integrity of the entire visual pathway. Visual information is transmitted between neurons at different levels in the retina by synapses. Photoreceptors, bipolar cells and ganglion cells are responsible for the major direct line of visual information flow in the retina. Horizontal cells and amacrine cells participate in feedback, integration and regulation of visual information in the lateral pathway, modulating the activity of the direct pathway of transmission through synaptic contacts in the inner plexiform layer (IPL) and outer plexiform layer (OPL), the two synaptic layers of the retina. Neurotransmitters play a role in synaptic activity in retinal neurons. There are a variety of neurotransmitters in the retina, including glutamate, GABA, glycine, acetylcholine, and so on. Glutamate and GABA are the major excitatory and inhibitory neurotransmitters in the retina, and they participate in the transmission and modulation of visual information.

In our previous study, we found that synapses in the retina can undergo plastic changes following acute high intraocular pressure (HIOP), in which the expression of the presynaptic marker synaptophysin (SYN) shows a distinct spatiotemporal pattern. Specifically, within 1 day after HIOP, the expression of SYN in the IPL increased slightly, and after 1 day, the distribution of SYN in the OPL widened and spread into the inner part of the outer nuclear layer (ONL), in which bouton-like vesicle-containing structures were observed by electron microscopy [3, 15]. Clarifying what types of neurotransmitters are involved in this process would help us to understand retinal synaptic plasticity after HIOP and ascertain its mechanisms and
potential intervention measures in the future.

In the present study, we detected the expression of glutamate and GABA following acute HIOP and further investigated whether glutamate or GABA, the major excitatory and inhibitory neurotransmitters in the retina, respectively, participated in the synaptic plasticity induced by acute HIOP.

II. Materials and Methods

Experimental animals
Forty-five healthy adult Sprague-Dawley rats of equal gender ratio weighing 200–250 g were obtained in-house from the animal center of Central South University, China (Permission No. SCKK (Xiang) 2009-0004). All of the animals were given tap water and food in an environmentally controlled room at a temperature of 20–24°C and a relative humidity of 55±10%, with a 12-hr light-dark cycle (lights on 6:00–18:00). All of the protocols for animal use complied with the “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985).

Establishment of acute HIOP models
The animal model of acute HIOP was established as previously described [1]. Briefly, animals were anesthetized by an intraperitoneal injection of 1:1 mixed 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. The intraocular pressure of all left eyes was gradually elevated to 110 mmHg and maintained for 60 min and then slowly descended to the normal level. A drop of chloramphenicol eye drop solution was administered to the conjunctival sac for infection prevention. The animals were allowed to survive for 2 hr, 6 hr, 12 hr, 1 day, 3 days, 7 days or 14 days. For the sham surgery group, the needle was inserted into the anterior chamber without elevating the pressure. Five rats with each time point were placed on the same slide.

Retinal tissue preparation
All of the rats were sacrificed at specific time points using deep anesthesia with excessive chloral hydrate (0.5 ml/100 g) administered by intraperitoneal injection. The rats were transectionally perfused with 0.9% sodium chloride followed by 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4). After perfusion, the eyeballs were dissected out, the cornea and lens were removed and the eyecups were post-fixed in the same fixative for 2 hr at room temperature. The eyecups were gradually dehydrated by immersion in sequential sucrose solution gradients (15% and 30%, respectively, in 0.1 M PB for 4 hr and overnight, respectively) at 4°C. The eyecups were subsequently embedded in Tissue-Tek optimal cutting temperature medium. Using a micrometer (Thermo Electron Corporation, Cheshire, UK), 14-µm-thick cryosections were obtained, and the sections containing the optic nerve were mounted on gelatin-coated slides and stored at −20°C until use. To minimize methodological variations, control and the experimental sections of each time point were placed on the same slide.

Immunofluorescent histochemistry
The slides were thawed and washed three times for 10 min with 0.01 M phosphate-buffered saline (PBS) containing 0.1% Tween-20. Subsequently, the sections were treated with blocking solution in 5% normal bovine serum in 0.1 M PB/0.3% Triton X-100 for 1 hr to block any nonspecific binding of the antibodies. Then, the sections were incubated with a mouse anti-SYN monoclonal antibody (1:8000, Sigma-Aldrich, St. Louis, MO) and a rabbit anti-glutamate polyclonal antibody (1:100, Millipore, Darmstadt, Germany) for 3 days at 4°C or a mouse anti-SYN monoclonal antibody (1:8000, Sigma-Aldrich, St. Louis, MO, USA) and a rabbit anti-GABA antibody (1:500, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. The sections were rinsed three times as described above. Subsequently, retinae were incubated for 2 hr at room temperature in the dark with Cy3-conjugated AffiniPure donkey anti-mouse IgG (1:400, Jackson Immuno Research, West Grove, PA, USA) and Alexa Fluor 488-conjugated AffiniPure donkey anti-rabbit IgG (1:400, Jackson Immuno Research, West Grove, PA, USA) and rinsed. Cell nuclei were stained for 8 min with Hoechst 33258 staining solution (Beyotime Institute of Biotechnology, Haimen, China). Finally, the sections were mounted in 1:1 0.1 M PB and glycerol (by volume) and stored at 4°C in the dark. A known positive antibody was used as a positive control. Negative control sections were prepared in exactly the same way, except that the primary antibody was replaced with 5% normal bovine serum. All images were captured using a laser scanning fluorescence microscope (Nikon 80i, Tokyo, Japan).

Image analysis
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 2560×1920 pixels (w×h) containing the designated area were selected for statistical analysis, and the distance along the retina was measured. The average gray value of glutamate and GABA immunoreactivity in the IPL was determined using Image J software. In this system, the gray value from black to white ranged from 0 to 255. The average gray value was negatively correlated with the intensity of positive products. The values of the experimental groups were compared with the normal control group (100%) on the same slide. Glutamate-positive cells in the ganglion cell layer (GCL), inner nuclear layer (INL) and ONL and GABA-positive cells in the INL were counted. Only cells that were clearly immunostained for glutamate or GABA and had a visible Hoechst 33258-stained nucleus were counted. The
number of positive cells was expressed as labeled neurons per linear millimeters of retina.

Statistical analysis

Measurement data were plotted as the mean±SEM. A one-way analysis of variance (one-way-ANOVA) was used to compare differences among groups with SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). The significance level was set at $p<0.05$.

III. Results

SYN expression in the retinae of rats with acute HIOP

In normal retinas, SYN immunoreactivity was prominent in the OPL and IPL with a punctate appearance, and increased staining was observed in the OPL (Figs. 1A1 and 3A1). The expression of SYN after HIOP induction showed a tendency of increasing from the IPL to the OPL, as reported in our previous study [3, 15]. The distribution of SYN did not change, and SYN immunofluorescence appeared slightly intense in the IPL within 1 day. From 3 days, SYN expression was increased in the IPL within 1 day and widened in the OPL after 1 day. A few glutamate-positive cells were observed in the GCL, INL and ONL in normal retina. Glutamate-positive cells were increased in the GCL at 6 hr and in the INL at 3–7 days and in the ONL at 3–7 days, which was in accordance with the tendency of SYN expression.
days to 14 days, SYN immunoreactivity was distributed throughout the entire IPL, but the IPL became progressively thinner because of injury. SYN expression and distribution in the OPL were similar to the normal control within 1 day after HIOP induction, but after 1 day, SYN immunoreactivity was gradually broadened and extended into the inner part of the ONL, reaching maximum distribution in the OPL and ONL on day 7 and then back to normal levels on day 14 post injury (Figs. 1 and 3). SYN immunoreactivity in the sham surgery group was similar to the normal group (images not shown).

**Glutamate expression in the retinae of rats with acute HIOP**

In normal retinae, a few glutamate-positive cells were observed in the GCL, INL and the ONL. Glutamate immunoreactivity was also observed in the IPL, in which glutamate colocalized with SYN (Fig. 1A2). The expression of glutamate following acute HIOP was as follows.

GCL: Glutamate-positive cells in the GCL were increased after 2 hr, with a peak at 6 hours ($p<0.05$) (Fig. 1C2). From 12 hr to 3 days, no significant change was detected in glutamate-positive cells in the GCL. Glutamate-positive cells in the GCL were markedly decreased after 7 days, with the death of retinal ganglion cells (RGCs) and the loss of inner retinal structure (Figs. 1 and 2A). IPL: Glutamate immunostaining in the IPL appeared more intense compared with the normal control between 3 days and 14 days after HIOP, with statistical

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**Fig. 2.** Quantification of glutamate immunoreactivity in rat retinae following acute HIOP. A shows the number of glutamate-positive cells located in the GCL, INL and ONL at different survival time after HIOP induction. The results are presented as the mean±SEM. Statistically significant differences were assessed according to a *one-way analysis of variance*. $\triangle$, * and #, represent the cell numbers in the GCL, INL and ONL, respectively, $p<0.05$ vs. normal control. B depicts the relative average gray value for glutamate-immunoreactive cells located in the IPL at different survival time after acute HIOP induction. The results are presented as the mean±SEM. Statistically significant differences were assessed with a *one-way analysis of variance*. $*, p<0.05$ vs. normal control. n: normal control. 2h, 6h, 12h, 1d, 3d, 7d, 14d represented 2, 6, 12 hours and 1, 3, 7, 14 days after HIOP, respectively.
significance \( p<0.05 \), although the IPL became thin in this period (Figs. 1 and 2B). INL: Glutamate-positive cells did not show a significant change in the INL within 1 day. The number of glutamate-positive cells in the INL was greatly increased after 1 day and reached the maximum on day 3, with some processes extending to the IPL. The number of glutamate-positive cells decreased slightly at 7–14 days but remained higher than the normal level (Figs. 1 and 2A; \( p<0.05 \)). ONL: Glutamate-positive cells were slightly increased in the ONL within 1 day and obviously increased at 3–7 days after acute HIOP, and this difference was statistically significant compared to the normal control (Figs. 1 and 2A; \( p<0.05 \)). The number of glutamate-positive cells was reduced to the normal level on day 14. Glutamate immunoreactivity in the sham surgery group was similar to the normal group (images not shown).

In summary, glutamate expression was increased from the inner part to the outer part of the retina following acute HIOP with survival time, which was the same as the pattern of SYN expression after HIOP.

Fig. 3. Fluorescence immunoreactivity of SYN (red) and GABA (green) in rat retinae of normal and HIOP. The nuclei of the cells were labeled with Hoechst (blue). A, A1, A2, A3: normal control; B, B1, B2, B3: 2 hr; C, C1, C2, C3: 6 hr; D, D1, D2, D3: 12 hr; E, E1, E2, E3: 1 day; F, F1, F2, F3: 3 days; G, G1, G2, G3: 7 days; H, H1, H2, H3: 14 days. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. In normal retina, a stratified distribution of GABA was observed in the IPL indicated by three bands. Mild GABA staining was also observed in the border of the OPL and a few GABA-positive neurons were in the INL. GABA distribution was similar to the normal following HIOP and was not directly correlated with SYN expression. Bar=20 \( \mu m \).
GABA expression in the retinæ of rats with HIOP

In normal retinæ, a punctate pattern of GABA staining was observed in the border of the OPL close to the INL and in the outer and inner portions of the IPL, and a weakly positive band was observed in the middle of the IPL (Fig. 3A2). There were a few GABA-positive neurons in the INL. At different survival time after HIOP induction, GABA immunoreactivity showed a similar pattern to the normal retina in the OPL. There was no apparent difference in the number of GABA-immunopositive cells in the INL between the retinæ of normal and HIOP rats (Figs. 3 and 4A). The distribution of GABA immunoreactivity in the IPL was also similar to the normal control, and the difference between the normal and HIOP groups in the relative average gray value for GABA in the IPL had no statistical significance (Figs. 3 and 4B; \( p>0.05 \)). Although SYN expression showed a spatiotemporal pattern from the IPL to the OPL after HIOP, GABA expression was not associated with this pattern (Fig. 3A3–H3). The expression of GABA in the sham surgery group was similar to the normal group (images not shown).

IV. Discussion

SYN is the most abundant integral membrane protein in synaptic vesicles at synaptic terminals and participates in

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**Fig. 4.** Quantification of GABA immunoreactivity in rat retinæ following acute HIOP induction. **A** indicates the number of GABA-positive cells located in the INL at different survival time after HIOP induction. The results are presented as the mean±SEM. Statistically significant differences were assessed with a one-way analysis of variance, *\( p<0.05 \) vs. normal control. **B** represents the relative average gray value for GABA-immunoreactive cells in the IPL at different survival time after acute HIOP induction. The results are presented as the mean±SEM. Statistically significant differences were assessed with a one-way analysis of variance, *\( p<0.05 \) vs. normal control. n: normal control. 2h, 6h, 12h, 1d, 3d, 7d, 14d represented 2, 6, 12 hours and 1, 3, 7, 14 days after HIOP, respectively.
the formation of synaptic vesicles and neurotransmitter release. SYN is often considered a marker of presynaptic terminals [13, 18]. SYN is a marker of synapse formation during neural tissue development and is also widely used in the study of synaptic plasticity in nervous system development [20, 34], injury [7, 16] and diseases [5, 29]. Our results were in accordance with a previous study [3, 15] in which SYN expression exhibited a spatiotemporal pattern that was initially successively increased from the IPL to the OPL and then returned to normal.

Glutamate is the major excitatory transmitter in the retina in the synaptic vesicles of nerve terminals and is principally expressed in photoreceptor cells, bipolar cells and ganglion cells in the retina, which directly participate in the transmission of visual signals [6, 31, 36]. Glutamate mediates excitatory neurotransmission via ionotropic NMDA-, AMPA- and kainate-type glutamate receptors and metabotropic glutamate receptors [8, 11, 12, 19]. Glutamate has also been implicated in the process of neuronal plasticity and survival in the developing visual system [26].

Extracellular glutamate has been shown to be elevated in the vitreous fluid of glaucoma patients [10] and in rat retinas under elevated IOP-induced ischemia [23, 27]. In the present study, we found that the expression of glutamate was gradually increased from the inner part to the outer part of the retina with the injury time. Following acute HIOP, RGCs and their axons suffered the most serious damage because they were closest to injury. The direct damage of axons in RGCs induced the interruption of visual signal transmission. Thus, RGCs attempted to increase synaptic transmission reactively, but because of damaged transmission, a large amount of neurotransmitters aggregated within the cells. Our experiment also detected that the number of glutamate-positive ganglion cells was increased at 6 hr post injury. We observed that the somata of glutamate-positive ganglion cells were large, which might be because large RGCs were more vulnerable to the stress of ocular hypertension [21, 35]. With the aggravation of retinal injury, these glutamate-positive large RGCs gradually died. Similar to ganglion cells, bipolar cells and photoreceptor cells (upstream of RGCs) were also exposed to the decrease in the transmission of visual signals through feedback signals in the retina and tried to compensate for the damaged visual transmission by increasing the synthesis of synaptic proteins, including SYN, to enhance synaptic transmission or generate new synapses. This result was in accordance with the results that SYN expression was increased internally to externally following acute HIOP, suggesting that glutamate might not participate in synaptic plasticity in the retina after acute HIOP. Alternatively, in the interval observed, the injury due to HIOP induction failed to cause obvious changes in GABA. Researchers have demonstrated that GABAergic neurons are relatively less sensitive than glycinergic amacrine cells in retinal ischemia/reperfusion models based on qPCR data [9]. Song G et al. found a large increase in the release of glutamate during initial experimental ocular hypertension in rabbits, while GABA was slightly but not significantly increased [32]. Moreno MC et al. also reported that glutamine uptake and release and glutaminase activity were significantly increased (p<0.01) in Wistar rats with ocular hypertension induced by hyaluronic acid for 3 weeks, but the distribution and intensity of GAD-immunoreactive cells did not differ between normal and hyaluronic acid-treated eyes [23, 24]. Our results were in agreement with these studies.

In conclusion, synapses in the retina might undergo plastic changes internally to externally following acute HIOP. The excitatory transmitter glutamate might participate in this process, but no evidence was found for a role of the inhibitory transmitter GABA.

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