Role of Glial Cells in Regulating Retinal Blood Flow During Flicker-Induced Hyperemia in Cats

Youngseok Song, Taiji Nagaoka, Takafumi Yoshioka, Seigo Nakabayashi, Tomofumi Tani, and Akitoshi Yoshida

Department of Ophthalmology, Asahikawa Medical University, Asahikawa, Japan

PurPOSE. To investigate how glial cells participate in retinal circulation during flicker stimulation in cats.

METHODS. Using laser Doppler velocimetry, we measured the vessel diameter and blood velocity simultaneously and calculated the retinal blood flow (RBF) in feline first-order retinal arterioles. Twenty-four hours after intravitreal injections of L-2-aminoadipic acid (LAA), a gliotoxic compound, and the solvent of 0.01 N hydrochloric acid as a control, we examined the changes in RBF in response to 16-Hz flicker stimulation for 3 minutes. We also measured the changes in RBF 2 hours after intravitreal injection of Nα-propyl-L-arginine (L-NPA), a selective neuronal nitric oxide synthase inhibitor, in LAA-treated eyes. To evaluate the effects of LAA on retinal neuronal function, ERGs were monitored. Immunohistochemical examinations were performed.

RESULTS. In LAA-treated eyes, histologic changes selectively occurred in retinal glial cells. There were no significant reductions in amplitude or elongation of implicit time in ERG after LAA injections compared with controls. In control eyes, the RBF gradually increased and reached the maximal level (53.5% ± 2.5% increase from baseline) after 2 to 3 minutes of flicker stimulation. In LAA-treated eyes, the increases in RBF during flicker stimulation were attenuated significantly compared with controls. In LAA-treated eyes 2 hours after injection of L-NPA, flicker-evoked increases in RBF decreased significantly compared with LAA-treated eyes.

CONCLUSIONS. The current results suggested that increases in RBF in response to flicker stimulation were regulated partly by retinal glial cells.

Keywords: retinal blood flow, flicker-induced hyperemia, retinal glial cells, gliotoxic compound, neurovascular coupling

To maintain neuronal function, the brain has evolved neurovascular coupling mechanisms to increase the regional blood flow, which Roy and Sherrington referred to as functional hyperemia, when they first described this concept more than a century ago. Retinal vessels dilate and retinal blood flow (RBF) increases as a result of the functional hyperemic response when the retina is stimulated by a flickering light, indicating that the retinal neural activity is associated with blood flow and metabolism, and considered as metabolic autoregulation in the retinal circulation.

Metabolic autoregulation in neurovascular coupling is maintained by three major cells (neurons, vasculature, and glial cells) in the brain and the retina. Glial cells, including Müller cells and astrocytes as the main glial cells in the retina, are vital for maintaining normal retinal function. Recent evidence from an animal experiment indicates that glial cells play a principal role in coupling neuronal activity to vessel dilation in retinal functional hyperemia. Indeed, impaired glial cell activity may be related to the pathologic mechanisms of ocular disorders such as diabetic retinopathy (DR) and glaucoma. Moreover, some clinical studies have reported that vasodilation of the retinal vessels elicited by flicker stimuli deteriorates in patients with these diseases. However, the involvement of glial cells in regulating the RBF in response to flicker stimulation has not been well determined. Although some reports have focused on the role of glial cells in metabolic autoregulation in neurovascular coupling, the role in the retinal vasculature remains unclear.

In previous studies, L-2-aminoadipic acid (LAA), a gliotoxic compound, was injected intravitreally to examine the specific role of glial cells in the retina. We investigated the role of retinal glial cells in regulating the RBF in response to flicker stimulation after suppressing the retinal glial cell function with intravitreal injection of LAA in cats.

MATERIALS AND METHODS

Animal Preparation

The Animal Care Committee of Asahikawa Medical University approved the study protocols in cats; the study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Thirty-six adult cats (2.6–3.2 kg) of either sex were tracheostomized and mechanically ventilated with room air containing 2% sevoflurane. The flow rate of sevoflurane was maintained at 1.5 L per minute during the experiment. Catheters were placed in the femoral arteries and vein. The mean arterial blood pressure (MABP) and heart rate (HR) were...
monitored continuously with a transducer (PowerLab; ADInstruments, Inc., Colorado Springs, CO, USA) and recorder (LabChart; ADInstruments, Inc.) in the proximal thoracic descending aorta. Pancuronium bromide (0.1 mg/kg per hour) (Daiichi Sankyo Co., Tokyo, Japan) was infused continuously via the femoral vein to maintain skeletal muscle relaxation. With the animal prone, the head was fixed in a stereotactic instrument. The arterial pH (pH), arterial partial carbon dioxide tension (PaCO2), arterial partial oxygen tension (PaO2), and bicarbonate ion (HCO3-) were measured intermittently with a blood gas analyzer (model AB15; Radiometer, Copenhagen, Denmark). The rectal temperature was measured and maintained between 37° and 38°C with a heated blanket. The pupils were dilated with 0.4% tropicamide (Santen Pharmaceuticals Co., Osaka, Japan). A 0-diopter contact lens (Seed Co., Ltd., Tokyo, Japan) was placed on the cornea, which was protected by instillation of a drop of sodium hyaluronate (Healon; Abbott Medical Optics, Inc., Abbott Park, IL, USA). A 26-gauge butterfly needle was inserted into the anterior chamber and connected to a pressure transducer and a balanced salt solution (Alcon, Fort Worth, TX, USA) reservoir for monitoring and maintaining the IOP at a constant level of 10 mm Hg, respectively. Table 1 shows the systemic and ocular parameters at rest.

### Retinal Blood Flow Measurements

A laser Doppler velocimetry (LDV) system (Laser Blood Flowmeter, model 100; Canon, Inc., Tokyo, Japan) customized for feline use was used to measure the retinal arteriolar diameter (D) (in micrometers) and velocity (V; mm/s) as described previously. The RBF in the arterioles (μL/min) was calculated based on the acquired V and D. Laser Doppler measurements of the temporal retinal arterioles were performed in one eye of each animal. The first-order arterioles described previously were stimulated by the flickering light and the RBF measurements were performed every 30 seconds during the stimulation period. The changes in the retinal circulatory parameters were expressed as the percent change from baseline. In the current study, because the blood flow reaches a plateau 2 minutes after flicker stimulation, in the current study, we expressed the average value of three points of 120 to 180 seconds as the maximal change. To assess whether LAA suppresses flicker-induced hyperemia in the retinal arterioles, we evaluated the changes in the RBF in response to flicker stimuli 24 hours after intravitreal injections of LAA or 0.01 N HCl as a control.

### Flicker Stimulation

As we showed previously, we used 16-Hz stimuli as flicker stimulation because the frequency obtained a maximal RBF response in cats and the eyes were allowed to dark-adapt for 2 hours before flicker stimuli. Fundus illumination was used only for alignment before dark adaptation started. The detailed protocol and instruments used in flicker stimulation were described previously.

### Intravitreal Injections and Chemicals

A 30-gauge needle (100-μL syringe; Hamilton, Reno, NV, USA) was used for the intravitreal injections (3 mm posterior to the limbus) with care taken not to injure the lens and retina. The head of the needle was positioned over the optic disc region. The LAA and bradykinin (BK) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). No-propyl-L-arginine (L-NPA) was obtained from Cayman Chemicals Co. (Ann Arbor, MI, USA). The drugs without LAA were dissolved in PBS. The LAA was dissolved in 0.01 N hydrochloric acid (HCl) because LAA does not dissolve in PBS. The volume of the intravitreal injections was 50 μL, which does not alter retinal circulatory parameters and minimizes the systemic effects of the inhibitors. Because the cat vitreous is approximately 2.5 mL, the 50-μL solution injected into the vitreous cavity is diluted by a factor of 50 near the retinal vessels. Hereafter, we refer to drug concentrations as injected concentrations.

The 20 and 60 mM LAA concentrations (final concentrations in the vitreous cavity of 0.4 and 1.2 mM, respectively) were chosen because 1.25 mM LAA causes swelling of the Müller cells and astrocytes while the remaining neural cells remain intact. The 24-hour time course after intravitreal injection of LAA was chosen because pathologic changes and dysfunction on the ERGs were not observed in the neural retina, although the Müller cells had some damage, that is, pale stained nuclei and increased glycogen granules 24 hours after the LAA injections.

### Changes in RBF to Flicker Stimulation

The measurements of D and V were started 5 minutes before flicker stimulation. The mean of five measurements at 1-minute intervals was recorded as the baseline value. The retina then was stimulated by the flickering light and the RBF measurements were performed every 30 seconds during the stimulation period. The changes in the retinal circulatory parameters were expressed as the percent change from baseline. In the current study, because the blood flow reaches a plateau 2 minutes after flicker stimulation, in the current study, we expressed the average value of three points of 120 to 180 seconds as the maximal change. To assess whether LAA suppresses flicker-induced hyperemia in the retinal arterioles, we evaluated the changes in the RBF in response to flicker stimuli 24 hours after intravitreal injections of LAA or 0.01 N HCl as a control.

### Effects of Gliotoxic Compound on Increased RBF in Response to BK

Because we confirmed previously that BK causes endothelium-dependent, nitric oxide (NO)-mediated vasodilation in isolated porcine retinal arterioles, we injected BK into the vitreous to cause the endothelium-dependent vasodilation. The increase in RBF induced by intravitreal injections of BK (50 μM) reached the maximal level at 120 minutes and persisted for at least 3 hours in our previous study. These concentrations were...
sufficient for the maximal vasodilation concentrations of BK, based on our previous in vitro study.²⁸

To assess the effect of LAA on endothelial vasodilatory function in the changes in the RBF in response to intravitreal injection of BK, the RBF was measured before and 2 hours after intravitreal injection of BK with pretreatment with LAA (20 mM, 60 mM) or 0.01 N HCl as a vehicle.

Changes in Basal Retinal Arterial Blood Flow Before and After Intravitreal Injection of the Gliotoxic Compound

To determine the effect of the gliotoxic compound on basal retinal circulation, we measured the basal RBF before and 24 hours after intravitreal injection of a 60-mM concentration of LAA (n = 4) in the same animals.

Effects of a Neuronal NO Synthase Inhibitor in LAA-Treated Eyes

We showed previously that increases in RBF during flicker stimulation were attenuated after intravitreal injection of L-NPA (5 mM), a selective neuronal NO synthase (nNOS) inhibitor,²⁶ suggesting that nNOS contributes to regulation of the retinal circulation during flicker stimulation. To determine whether the decrease in RBF in response to flicker stimulation in LAA (60 mM)-treated eyes resulted from reduced NO by nNOS in retinal glial cells, we measured the RBF in response to flicker stimulation in LAA (60 mM)-treated eyes 2 hours after intravitreal L-NPA injection (5 mM). The L-NPA was injected 22 hours after LAA to confirm the maximal responses of LAA and L-NPA.

Immunohistochemistry

For whole-mount assessment, the eyes were enucleated and fixed in 1% paraformaldehyde for 1 hour. The retina was blocked and permeabilized in 5% goat serum with 0.3% Triton X-100 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS for 1 to 2 hours. The retinas were transferred to primary antibodies diluted in block solution and incubated for 1 hour at room temperature. The primary antibody was mouse antigliarial fibrillary acidic protein (GFAP)-cy3 antibody (1:400; Sigma Chemical Co.) and isolectin IB4 conjugated to Alexa Fluor 647 (1:200) (Invitrogen, Carlsbad, CA, USA) was stained. The slides were mounted (Dako, Tokyo, Japan) and observed for green (cy3) and red (Alexa Fluor 647) staining and analyzed with a fluorescence microscope (Fluoview FV 1000; Olympus, Tokyo, Japan). Photoshop CS 6 (Adobe Systems, Inc., San Jose, CA, USA) was used to quantify the GFAP expression. The mean thickness of three sites (300 × 300 µm) for each group were selected randomly in the observed area approximately 3 mm superior to the center of the optic nerve head (ONH) and measured, and the averages were compared.

Histologic Examination

To determine the gliotoxic effects of LAA, histologic examinations were performed 24 hours after intravitreal injection of LAA 60 mM or 0.01N HCl. The enucleated eyes were fixed in 2% paraformaldehyde-2.5% glutaraldehyde in 10 mM PBS for 3 hours, then fixed in 4% paraformaldehyde, and embedded in paraffin. A transverse section of each retina (5 µm) was cut parallel to the medullary rays of the ONH. The section of the retina was stained with hematoxylin and eosin (HE) and examined by light microscopy. To evaluate the retinal neural damage, the thicknesses of the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) were measured in transverse sections. For this analysis, three light photomicrographs (magnification: ×400) were taken in a masked fashion approximately 3 mm superior to the center of the ONH. The thickness of each layer was averaged for each eye to obtain data for statistical analysis.

Electroretinogram Recording and Analysis

To determine the selective gliotoxicity of LAA on the retinal function, ERGs were performed before and 24 hours after intravitreal injection of LAA (60 mM) or 0.01 N HCl as a solvent. A light-emitting diode light stimulator (LS-C; Mayo Corporation, Aichi, Japan) and Ganzfeld Dome, a data acquisition system, and AC amplifier (PuREC system, PC-100; Mayo Corporation) were used to record the ERG. Before the ERG recordings, the cats were dark-adapted for 2 hours after mydriasis with 0.4% tropicamide (Santen Pharmaceutical Co.). The ERG was performed under general anesthesia induced by sevoﬂurane; recordings were performed with a gold ring active electrode on the cornea, a gold disk negative electrode in the mouth, and the ground electrode on an earlobe by single flash stimulation. The flash stimulus intensity was 1.0 cd·s/m². Bandpass filters were set at 0.3 to 500 Hz and the amplifier gain was set at ×10,000 for the a- and b-waves. The amplitudes and the implicit times of the a- and b-waves were measured. All waveforms were analyzed by the PuREC system. We performed ERGs before and 24 hours after intravitreal injection of LAA at 60 mM (n = 7) or 0.01 N HCl (n = 7) as a control, and the amplitudes and the implicit times of the a- and b-waves of LAA-treated eyes were compared with those of the controls.

Statistical Analysis

All data are expressed as the mean percentage ± SEM. The vasodilator responses were calculated as the percentage increases of the RBF from baseline. For statistical analysis, we used ANOVA for repeated measurements, followed by post hoc comparison with the Dunnett procedure. Group comparisons of the RBF, histologic examinations, and ERGs were performed using the Mann-Whitney U test or Wilcoxon signed-rank test. Values of P less than 0.05 were considered statistically significant.

RESULTS

Effects of Gliotoxic Compound on RBF at Baseline and in Response to Intravitreal Injection of BK

Twenty-four hours after injection of LAA (60 mM), there were no significant changes in retinal (D, V, RBF) and systemic circulatory (pH, PaCO2, PaO2, HCO3⁻, MABP, or HR) parameters (n = 6) (Table 2) or in the amplitude or elongation of the implicit time of the ERG a- and b-waves (n = 7) (Table 3).

In the 20- and 60-mM LAA groups, increases in D, V, and RBF induced by intravitreal injection of BK were comparable to those in the control groups (Fig. 1). There were no significant changes in any systemic circulatory parameters (pH, PaCO2, PaO2, HCO3⁻, MABP, or HR) before and 120 minutes after intravitreal injection of BK (data not shown).

Effects of Gliotoxic Compound on RBF in Response to Flicker Stimulation

After 3 minutes of 16-Hz flicker stimulation, the D, V, and RBF maximally increased by 5.9% ± 1.2%, 36.9% ± 2.1%, and 55.5% ± 2.5%, respectively, in the control group. In the LAA groups, those changes were significantly lower, the D, V, and
The data are the actual measured values. Each data point (before and after) was measured in the same individual. Data are expressed as the means ± SEM. To compare before with after injections of LAA (60 mM), we used the Wilcoxon signed-rank test. \( P < 0.05 \) was considered significant. There are no significant (\( P > 0.05 \)) differences in any parameters between before and after injections. Before indicates before the intravitreal injections; after indicates 24 hours after the intravitreal injections.

### Effects of nNOS Inhibitor L-NPA on Flicker-Evoked Increase in Retinal Circulation in LAA-Treated Eyes

In LAA-treated eyes, intravitreal injection of L-NPA (5 mM) significantly reduced the flicker-induced increases in RBF compared with eyes treated with only LAA (Fig. 3). Before and 2 hours after injection of L-NPA and during flicker stimulation, the systemic parameters (pH, PaCO\(_2\), PaO\(_2\), MABP, or HR) did not change significantly, respectively, in the 60-mM LAA group (data not shown). In the preliminary study, we confirmed the absence of significant differences in the increases in the RBF in response to the flicker stimuli between the 0.01 N HCl- and PBS-treated eyes (data not shown).

### Immunohistochemistry

To examine the effect of LAA, GFAP staining was assessed in the whole retina (Fig. 5). GFAP immunofluorescence histochemistry (green astrocytes) and binding of isoelectric Ib4 (red vessels) was performed on flat-mounted feline retinal preparations before (Fig. 5A) and 24 hours after intravitreal injection of 60 mM of LAA (Fig. 5B). Compared with the control, mean densities of GFAP expression were significantly (\( P < 0.05 \)) reduced in LAA-treated eyes (Table 5).

### Discussion

Many studies have reported that glial cells contribute to neurovascular coupling in the brain.\(^5,30-35\) Metea and Newman\(^6\) reported that glial cells may contribute to neurovascular coupling in the rat ex vivo retina. In that study, selective stimulation of glial cells resulted in both vasodilation and vasoconstriction, and light-evoked vasodilation was blocked when the purinergic antagonist suramin interrupted neuronal-to-glial signaling.\(^5\) In the current study, although we did not observe decreases in the RBF, we showed that the increases in RBF during flicker stimulation were attenuated significantly by intravitreal injection of LAA compared with the control (Fig. 2).

### Table 2. Systemic and Ocular Parameters Before and 24 Hours After Intravitreal Injection of LAA at 60 mM (\( n = 4 \))

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.42 ± 0.01</td>
<td>7.40 ± 0.01</td>
</tr>
<tr>
<td>PaCO(_2), mm Hg</td>
<td>27.2 ± 1.1</td>
<td>28.6 ± 1.0</td>
</tr>
<tr>
<td>PaO(_2), mm Hg</td>
<td>114.5 ± 2.6</td>
<td>111.8 ± 4.1</td>
</tr>
<tr>
<td>HCO(_3)^(-), mM</td>
<td>20.7 ± 0.7</td>
<td>20.1 ± 0.3</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>105.2 ± 1.0</td>
<td>103.5 ± 1.3</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>121.0 ± 1.4</td>
<td>120.3 ± 4.3</td>
</tr>
<tr>
<td>OPP, mm Hg</td>
<td>93.6 ± 0.9</td>
<td>91.7 ± 1.4</td>
</tr>
<tr>
<td>Diameter, μm</td>
<td>110.6 ± 2.9</td>
<td>110.7 ± 4.0</td>
</tr>
<tr>
<td>Velocity, mm/s</td>
<td>31.1 ± 3.6</td>
<td>34.6 ± 2.9</td>
</tr>
<tr>
<td>RBF, μL/min</td>
<td>9.0 ± 1.3</td>
<td>10.2 ± 1.4</td>
</tr>
</tbody>
</table>

Table 4. There were no significant (\( P > 0.05 \)) differences in the thickness in each layer between the LAA-treated and control eyes.

### Table 3. Effect of LAA on ERGs (\( n = 7 \))

<table>
<thead>
<tr>
<th>Type</th>
<th>Control</th>
<th>LAA, 60 mM</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-wave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Amplitude</td>
<td>103.2 ± 4.6</td>
<td>99.0 ± 5.2</td>
<td>0.38</td>
</tr>
<tr>
<td>Implicit time</td>
<td>102.5 ± 4.2</td>
<td>99.6 ± 4.4</td>
<td>0.74</td>
</tr>
<tr>
<td>b-wave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Amplitude</td>
<td>96.0 ± 5.0</td>
<td>105.1 ± 6.5</td>
<td>0.52</td>
</tr>
<tr>
<td>Implicit time</td>
<td>100.5 ± 3.1</td>
<td>101.9 ± 3.6</td>
<td>0.45</td>
</tr>
</tbody>
</table>

### Table 4. Thicknesses of the GCL, INL, and ONL Before and 24 Hours After Intravitreal Injection of LAA (60 mM) (\( n = 5 \))

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control</th>
<th>LAA, 60 mM</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCL, μm</td>
<td>16.1 ± 0.8</td>
<td>15.9 ± 0.9</td>
<td>0.59</td>
</tr>
<tr>
<td>INL, μm</td>
<td>17.8 ± 0.5</td>
<td>19.4 ± 0.7</td>
<td>0.16</td>
</tr>
<tr>
<td>ONL, μm</td>
<td>46.6 ± 2.2</td>
<td>50.7 ± 3.0</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM for five cats. \( P \) values obtained by Mann-Whitney U test are shown. There are no significant (\( P > 0.05 \)) differences in any layers between groups.
Although recent ex vivo animal studies have reported that the retinal glial cells may play a principal role in functional hyperemia,\textsuperscript{6,16} it is unclear whether glial cells regulate basal blood flow in the in vivo retina. In the current study, there was no significant difference in the basal RBF between before and after intravitreal injection of LAA (Table 2). Although the current findings cannot fully answer the question, there are three plausible reasons for this result. First, glial cells do not help regulate basal blood flow in the retina; second, the retina has a compensatory mechanism for regulating basal blood flow after suppressed glial function; and third, the retinal glial cells are partially blocked by LAA at a concentration of 60 mM so that the basal RBF does not change. Further studies are needed to clarify whether retinal glial cells help regulate basal blood flow.

Previous studies have shown that NO plays an important role in flicker-induced vasodilation in animals\textsuperscript{4} and humans.\textsuperscript{36} Recently, we reported that L-NPA (5 mM), a selective nNOS inhibitor, reduced flicker-induced increases in RBF by a third of the baseline value in cats, meaning that two-thirds of the flicker-induced hyperemia is generated by NO by nNOS in neuronal and/or glial cells in the retina.\textsuperscript{26} The current study confirmed that LAA (60 mM) as a gliotoxic compound reduced flicker-induced increases in the RBF by a third of the baseline value, indicating that two-thirds of the flicker-induced hyperemia is generated by the retinal glial cells. In addition, a flicker-

**FIGURE 2.** Time course of the changes from baseline in retinal circulation in response to flicker stimulation in the following groups: 0.01N HCl (n = 6) as a control, 20 mM of LAA (n = 6), and 60 mM of LAA (n = 6). (A) The black bar represents the period of flicker (3 minutes) (frequency 16 Hz, modulation depth 100%, dark adaptation time 2 hours). The data are expressed as the mean percentage ± SEM of baseline. *P < 0.05 and †P < 0.05 compared with a control group by 2-way repeated-measures ANOVA followed by the Dunnett procedure. (B) Maximal changes from baseline in the retinal circulation in response to flicker stimulation in (A). The data are expressed as the mean percentage ± SEM of baseline. *P < 0.05 compared with a control group by 1-way factorial ANOVA followed by the Dunnett procedure.

**TABLE 5.** Mean Densities (pixels/mm) of GFAP Expression in the Retina

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LAA, 60 mM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean density</td>
<td>184305.5 ± 26000.2</td>
<td>27675.6 ± 3190.5</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The mean densities of GFAP expression in astrocytes were compared quantitatively between two groups. Compared with the control, the mean densities of GFAP expression are significantly (P = 0.03) reduced in LAA-treated eyes analyzed using the Mann-Whitney U test. Data are expressed as mean ± SEM in two groups.
induced increase in RBF was abolished by double blocking with L-NPA (5 mM) and LAA (60 mM). These results suggested that flicker-induced hyperemia may be generated by three prominent vasodilatory factors: NO by the nNOS in neurons, NO by the nNOS in glial cells, and another vasodilatory factor in glial cells (Fig. 5). Indeed, some studies have reported that nNOS protein was expressed in neurons and glial cells in mammalian retina.37–42 Moreover, in retinal functional hyperemia, there are several vasodilatory candidates in glial cells without NO from glial cells, such as prostaglandin E2 (PGE2) and epoxyeicosatrienoic acids (EETs).32 The current study did not confirm definitively that the retinal glial cells play a dominant role (at least accounting for two-thirds of the regulation in flicker-induced hyperemia) in regulating flicker-induced hyperemia in the retina. Further studies should determine the role of vasodilatory factors other than NO in glial cells in regulating flicker-induced hyperemia in the retina.

It is well known that pericytes cover a large fraction of the capillary surface in the brain. Vasoactive molecules, such as NO, PGE_2, or EETs, in astrocytes may cause pericytic dilation in capillaries and increase cerebral blood flow in functional hyperemia.43 In the retina, pericytes cover the capillary surface more extensively than in the brain,44 indicating that the interaction between glial cells and pericytes may be more important in functional hyperemia in the retina than the brain. Moreover, Kornfield and Newman45 reported that flicker-evoked vasodilation depended on vessel size and depth in the retina. Indeed, both pericytic loss and impaired glial activity in the retina are detected before DR appears clinically and then the diminished response in flicker-evoked vasodilation is observed.46,47 In the current study, suppression of the

![Figure 4](image-url) - Photomicrographs of transverse sections of the retina stained with HE. Each section was obtained from eyes with intravitreal injection of 0.01 N HCl as a control (A) and LAA at 60 mM. No apparent changes are observed in the GCL, INL, and ONL of the retina. Scale bar: 50 μm.

![Figure 5](image-url) - Immunohistochemistry of flat-mounted retina. Astrocytes are stained with anti-GFAP antibody (green) and vessels are stained with lectin IB4 (red). The retinas were dissected from eyes given an intravitreal injection of 0.01N HCl as a control (A) and LAA at a concentration of 60 mM (B). Compared with the control (A), the astrocytic processes in LAA-treated eyes (B) seem shorter and deformed. Scale bars: 50 μm.
flicker-evoked increase in blood V was greater compared with that in vessel D in LAA-treated eyes. Because blood V in the retinal arterioles measured by LDV may reflect the entire hemodynamics in the retinal vasculature including the downstream arterioles and capillaries, the current findings suggested that capillaries and not retinal arterioles may be primarily responsible for controlling the retinal circulation in flicker-induced hyperemia in the retina, which was mediated by glial cell activity.

In the present study, there were no significant changes in the implicit time of the ERG a- and b-waves after intravitreal injection of LAA at a concentration of 60 mM (Table 3). Although Welinder et al. reported that the amplitude of the ERG b-wave decreased in rabbit eyes injected with LAA, they used a higher concentration of LAA (150 mM) than in our study. Indeed, another previous report showed that LAA at a concentration of 200 mM caused neural damage and significantly affected the ERG b-wave at 24 hours in rabbits. In our preliminary experiment, we also confirmed that LAA at a concentration of 200 mM caused a significant reduction in the amplitude of the ERG b-wave in cats (data not shown). However, some reports have suggested that the implicit time of the ERG b-wave was not prolonged significantly after injections of low concentrations of LAA despite changes in the Müller cells and astrocytes. In fact, a recent immunohistochemistry evaluation found that LAA at a concentration of 60 mM damaged the retinal glial cells, histologic evaluation showed intact neural cells, and there was no significant reduction of the ERG b-wave. In addition, we also determined whether 60 mM of LAA injected intravitreally may have any toxic effects on the neurons, which was confirmed by ERG and histologic examination in the current study. Despite the morphologic changes in the glial cells after LAA (Fig. 5; Table 5), the implicit time and amplitudes of the ERG a- and b-waves (Table 3) and the thickness of the GCL, INL, and ONL (Table 4) did not change significantly after intravitreal injection of a 60-mM concentration of LAA compared with the control. In addition, the increase in RBF induced by BK, which elicits endothelium-dependent, NO-mediated vasodilation in isolated porcine retinal arterioles, did not change significantly after intravitreal injection of both concentrations of LAA (Fig. 1). These results suggested that LAA concentrations in the current study selectively damaged glial cells without hurting the neurons and retinal vasculature.

The current study had some limitations. First, we did not clarify the detailed molecular mechanism of how retinal glial cells regulate RBF in response to flicker stimulation. Although we recently found possible involvement of the retinal NO derived from nNOS in flicker-induced hyperemia in the retina in anesthetized cats, the current findings that L-NPA further reduced flicker-induced hyperemia in the retina after LAA treatment may indicate that NO derived from nNOS in retinal neurons and retinal glial cells may be involved in the flicker-induced hyperemia in the retina. In addition, although it was suggested that one particular mechanism of neurovascular coupling in which glial cells release vasodilatory PGE2 and/or EETs as the arachidonic acid metabolites produced by cyclooxygenase (COX) is a principal and perhaps dominant mechanism mediating functional hyperemia in the retina, we did not examine the role of these molecules in flicker-induced hyperemia in the retina because there is no selective and specific blocker of COX that is only in the retinal glial cells. Second, the current results did not determine which retinal glial cells, the Müller glial cells or astrocytes, play a central role in retinal circulation in response to flicker stimulation, because it is difficult to suppress separately each cellular function using a gliotoxic compound that affects both of them. Third, although the current data did not provide a definitive explanation for the effect of general anesthesia, we previously found in a preliminary study that sevoflurane per se did not change the vessel D of isolated porcine retinal arterioles (data not shown) and the changes in the concentration of...
ocular blood flow and decreases in flicker-evoked retinal adduction, these ocular diseases also have impaired regulation of the future.

Impaired glial cellular activity is related to the pathogenesis of some ocular diseases, such as DR or glaucoma. In addition, these ocular diseases also have impaired regulation of ocular blood flow and decreases in flicker-evoked retinal vasodilation. Although it is unclear whether glial cell dysfunction or insufficiency of the retinal circulation is the initial pathogenetic event in these diseases, dysfunction of the retinal glial cells participates in progression in these diseases. Therefore, further basic and clinical studies are warranted to examine whether improved glial function may be a novel target for treating ocular vascular disorders.

In conclusion, we found for the first time that the flicker-induced hyperemia in the retina was decreased in LAA-treated eyes, suggesting that glial cells play a major role in regulating RBF in response to flicker stimulation. Our findings indicated that three prominent types of vasodilators (i.e., nNOS from neurons, nNOS from glial cells, and other vasodilatory factors from glia) may contribute to the phenomena (Fig. 6). Because it has been reported previously that glial cell dysfunction may be involved in the pathogenesis of DR and glaucoma, clarifying the detailed mechanisms of glial cells in the retinal vasculature may provide a further understanding of the pathogenesis in these ocular disorders.

Acknowledgments

Supported by a Grant-in-Aid for Scientific Research (B) 25293352 and Challenging Exploratory Research 25670724 from the Ministry of Education, Science, and Culture, Tokyo, Japan (TN) and a Grant-in-Aid for Scientific Research (B) 26861430 from the Ministry of Education, Science, and Culture, Tokyo, Japan (YS). The authors alone are responsible for the writing and the content of the paper.

Disclosure: Y. Song, None; T. Nagoaka, None; T. Yoshioka, None; S. Nakabayashi, None; T. Tani, None; A. Yoshida, None

References

29. Sagawa K, Nagaoka T, Izumi N, Nakabayashi S, Yoshida A. Acute hyperglycemia-induced endothelial dysfunction in...


44. Frank RN, Dutta S, Mancini MA. Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. *Invest Ophtalmol Vis Sci.* 1987;28:1086–1091.


