Investigative Ophthalmology and Visual Science (2015.11) 56(12):7551-7559.

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Running title: Glial Cell Role in Retinal Blood Flow

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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 (to TN) and a Grant-in-Aid for Scientific Research (B) 26861430 from the Ministry of Education, Science, and Culture, Tokyo, Japan (to YS)

Word count: 4,259 words

The authors have no financial/conflicting interests to disclose.

ABSTRACT

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 Nω-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, electroretinograms (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

Précis

The reduction in the flicker-evoked increase in retinal circulation after intravitreal injection of gliotoxic compound in cats suggests that retinal glial cells are involved in the regulation of the retinal circulation during flicker stimulation.

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.[1,2] 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.[3,4]

Metabolic autoregulation in neurovascular coupling is maintained by three major cells, i.e., neurons, vasculature, and glial cells, in the brain and the retina.[2,5,6] Glial cells, including Müller cells and astrocytes as the main glial cells in the retina,[7] are vital for maintaining normal retinal function.[8] 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.[6] Indeed, impaired glial cell activity may be related to the pathological mechanisms of ocular disorders such as diabetic retinopathy (DR) and glaucoma.[9-12] Moreover, some clinical studies have reported that vasodilation of the retinal vessels elicited by flicker stimuli deteriorates in patients with these diseases.[13-15] However, the involvement of glial cells in regulating the RBF in response to flicker stimulation has not been well determined.[16] Although some reports have focused on the role of glial cells in metabolic autoregulation in neurovascular coupling,[2,6,16-18] the role in the retinal vasculature remains unclear.

In previous studies, L-2-aminoadipic acid (LAA),[17,19,20] 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/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) and recorder (LabChart, ADInstruments Inc.) in the proximal thoracic descending aorta. Pancuronium bromide (0.1 mg/kg/h) (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 stereotaxic instrument. The arterial pH (pH), arterial partial carbon dioxide tension ($PaCO_2$), arterial partial oxygen tension (PaO_2), and bicarbonate ion ($HCO3^-$) were measured intermittently with a blood gas analyzer (model ABL5, 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 Pharmaceutical 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). 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) reservoir for monitoring and maintaining the intraocular pressure (IOP) at a constant level of 10 mmHg, respectively. Table 1 shows the systemic and ocular parameters at rest.

RBF 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) (millimeters/second) as described previously.[21,22] The RBF in the arterioles (microliters/minute) 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 were chosen for study because they have relatively straight segments and were sufficiently distant from the adjacent vessels for consistent measurements.

The RBF was calculated using the formula $RBF = S \times V_{mean}$, where S is the cross-sectional area of the retinal arteriole at the laser Doppler measurement site, assuming a circular cross-section, and V_{mean} is the mean blood V calculated as $V_{mean} = V_{max}/2$.[23] The MABP was determined using the formula MABP = diastolic BP + (systolic BP - diastolic BP)/3, which is the index of the systemic BP. Because the cats were prone during the experiments, the ocular perfusion pressure (OPP) was calculated as OPP = MABP – IOP.[24,25]

Flicker Stimulation

As we showed previously,[26] 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.[26] Fundus illumination was used only for

alignment before dark adaptation started. The detailed protocol and instruments used in flicker stimulation were described previously.[26]

Intravitreal Injections and Chemicals

A 30-gauge needle (100-µL syringe; Hamilton, Reno, NV) was used for the intravitreal injections (3 mm posterior to the limbus) with care taken not to injure the lens and retina.[21] The head of the needle was positioned over the optic disc region. LAA and bradykinin (BK) were purchased from Sigma Chemical Co. (St. Louis, MO). Nω-propyl-L-arginine (L-NPA) was obtained from Cayman Chemicals Co. (Ann Arbor, MI). The drugs without LAA were dissolved in phosphate buffered saline (PBS). 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.[21] Because the cat vitreous is about 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 L-2-aminoadipic acid causes swelling of the Müller cells and astrocytes while the remaining neural cells remain intact.[27] The 24-hour time course after intravitreal injection of LAA was chosen because pathologic changes and dysfunction on the electroretinograms (ERGs) were not observed in the neural retina, although the Müller cells had some damage, i.e., pale stained nuclei and increased glycogen granules 24 hours after the LAA injections.[17,27]

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.[26] 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,[28] we injected BK into the vitreous to cause the endothelium-dependent vasodilation.[29] 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.[29] 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 pre-treatment 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 nNOS 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). 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 (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 anti-glial fibrillary acidic protein (GFAP)-cy3 antibody (1:400; Sigma-Aldrich, St. Louis, MO) and isolectin IB4 conjugated to Alexa Fluor 647 (1:200) (Invitrogen, Carlsbad, CA) was stained.Ok as changed? 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., Tokyo) was used to quantify the GFAP expression. The mean densities of three sites (300 × 300 μm) for each group were selected randomly in the observed area about 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 performed24 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 about 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.

ERG 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 ERGs. 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 dish negative electrode in the mouth, and the ground electrode on an earlobe by single flash stimulation. The flash stimulus intensity was 1.0 cds/m^2. Bandpass filters were set at 0.3 to 500 Hz and the amplifier gain was set at x10,000 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 ± standard error of the mean. The vasodilator responses were calculated as the percentage increases of the RBF from baseline. For statistical analysis, we used analysis of variance (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. $P < 0.05$ was 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, $PaCO_2$, PaO_2, 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, $PaCO_2$, PaO_2, 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 53.5 ± 2.5%, respectively, in the control group. In the LAA groups, those changes were significantly lower; the D, V, and RBF maximally increased by 4.5 ± 1.0%, 25.4 ± 3.5%, and 37.1 ± 5.4%, respectively, in the 20-mM LAA group maximally increased by 2.8 ± 0.8%, 12.1 ± 3.3%, and 19.6 ± 2.4%, respectively, in the 60-mM LAA group (Fig. 2). There were no significant changes in any systemic parameters (pH, $PaCO_2$, PaO_2, HCO3-, MABP, or HR) before, during, and after flicker stimulation (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).

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, HCO3-, MABP, or HR) did not change significantly (data not shown).

Histologic Examination

Fig. 4 shows transverse retinal sections stained with HE. There were no apparent changes in morphology in the GCL, INL, or ONL in the LAA-treated eyes. A quantitative assessment of the effect of LAA on the GCL, INL, and ONL is shown in Table 4. There were no significant ($P > 0.05$) differences in the thickness in each layer between the LAA-treated and control eyes.

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 isolectin 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.[30-35] Metea and Newman reported that glial cells may contribute to neurovascular coupling in the rat ex vivo retina.[6] 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.[6] In the current study, though 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). These conflicting results may be due to differences in experimental methodology, i.e., the current study was an in vivo experiment in cats, whereas the previous report used ex vivo whole-mount rat retina. However, both studies clearly showed that retinal glial cells regulate RBF during flicker stimulation.

Although recent ex vivo animal studies have reported that the retinal glial cells may play a principal role in functional hyperemia,[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[4] and humans.[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.[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

318 flicker-induced increase in RBF was abolished by double blocking with L-NPA (5 mM)
319 and LAA (60 mM). These results suggested that flicker-induced hyperemia may be
320 generated by three prominent vasodilatory factors: NO by the nNOS in neurons, NO by
321 the nNOS in glial cells, and another vasodilatory factor in glial cells (Fig. 5). Indeed,
322 some studies have reported that nNOS protein was expressed in neurons and glial cells
323 in mammalian retina.[37-42] Moreover, in retinal functional hyperemia, there are several
324 vasodilatory candidates in glial cells without NO from glial cells, such as prostaglandin
325 E_2 (PGE_2) and epoxyeicosatrienoic acids (EETs).[32] The current study did not confirm
326 definitively that the retinal glial cells play a dominant role (at least accounting for
327 two-thirds of the regulation in flicker-induced hyperemia) in regulating flicker-induced
328 hyperemia in the retina. Further studies should determine the role of vasodilatory factors
329 other than NO in glial cells in regulating flicker-induced hyperemia in the retina.
330 It is well known that pericytes cover a large fraction of the capillary surface in the
331 brain. Vasoactive molecules such as NO, PGE_2, or EETs in astrocytes may cause
332 pericytic dilation in capillaries and increase cerebral blood flow in functional
333 hyperemia.[43] In the retina, pericytes cover the capillary surface more extensively than in
334 the brain,[44] indicating that the interaction between glial cells and pericytes may be more
335 important in functional hyperemia in the retina than the brain. Moreover, Kornfield and
336 Newman reported that flicker-evoked vasodilation depended on vessel size and depth in
337 the retina.[45] Indeed, both pericytic loss and impaired glial activity in the retina are
338 detected before DR appears clinically and then the diminished response in
339 flicker-evoked vasodilation is observed.[46,47] In the current study, suppression of the
340 flicker-evoked increase in blood V was greater compared with that in vessel D in
341 LAA-treated eyes. Because blood V in the retinal arterioles measured by LDV may

342 reflect the entire hemodynamics in the retinal vasculature including the downstream
343 arterioles and capillaries, the current findings suggested that capillaries and not retinal
344 arterioles may be primarily responsible for controlling the retinal circulation in
345 flicker-induced hyperemia in the retina, which was mediated by glial cell activity.

346 In the present study, there were no significant changes in the implicit time of the
347 ERG a- and b-waves after intravitreal injection of LAA at a concentration of 60 mM
348 (Table 3). Although Welinder et al. reported that the amplitude of the ERG b-wave
349 decreased in rabbit eyes injected with LAA[48], they used a high concentration of LAA
350 (150 mM) greater than in our study. Indeed, another previous report showed that LAA
351 at a concentration of 200 mM caused neural damage and significant affected the ERG
352 b-wave at 24 hours in rabbits.[17] In our preliminary experiment, we also confirmed that
353 LAA at a concentration of 200 mM caused a significant reduction in the amplitude of
354 the ERG b-wave in cats (data not shown). However, some reports have suggested that
355 the implicit time of the ERG b-wave was not prolonged significantly after injections of
356 low concentrations of LAA despite changes in the Muller cells and astrocytes.[19,20] In
357 fact, a recent immunohistochemistry evaluation found that LAA at a concentration of 60
358 mM damaged the retinal glial cells, histologic evaluation showed intact neural cells, and
359 there were no significant reduction of the ERG b-wave.[17] In addition, we also
360 determined whether 60 mM of LAA injected intravitreally may have any toxic effects
361 on the neurons, which was confirmed by ERG and histologic examination in the current
362 study. Despite the morphologic changes in the glial cells after LAA (Fig. 5, Table 5), the
363 implicit time and amplitudes of the ERG a- and b-waves (Table 3) and the thickness of
364 the GCL, INL, and ONL (Table 4) did not change significantly after intravitreal
365 injection of a 60-mM concentration of LAA compared with the control. In addition, the

366 increase in RBF induced by BK, which elicits endothelium-dependent, NO-mediated
367 vasodilation in isolated porcine retinal arterioles,[28] did not change significantly after
368 intravitreal injection of both concentrations of LAA (Fig. 1). These results suggested
369 that LAA concentrations in the current study selectively damaged glial cells without
370 hurting the neurons and retinal vasculature.

371 The current study had some limitations. First, we did not clarify the detailed
372 molecular mechanism of how retinal glial cells regulate RBF in response to flicker
373 stimulation. Although we recently found possible involvement of the retinal NO derived
374 from nNOS in flicker-induced hyperemia in the retina in anesthetized cats,[26] the current
375 findings that L-NPA further reduced flicker-induced hyperemia in the retina after LAA
376 treatment may indicate that NO derived from nNOS in retinal neurons and retinal glial
377 cells may be involved in the flicker-induced hyperemia in the retina. In addition,
378 although it was suggested that one particular mechanism of neurovascular coupling in
379 which glial cells release vasodilatory PGE_2 and/or EETs as the arachidonic acid
380 metabolites produced by cyclooxygenase (COX) is a principal and perhaps dominant
381 mechanism mediating functional hyperemia in the retina,[5] we did not examine the role
382 of these molecules in flicker-induced hyperemia in the retina because there is no
383 selective and specific blocker of COX that is only in the retinal glial cells. Second, the
384 current results did not determine which retinal glial cells, the Müller glial cells or
385 astrocytes, play a central role in retinal circulation in response to flicker stimulation,
386 because it is difficult to suppress separately each cellular function using a gliotoxic
387 compound, which affects both of them.[19,20] Third, although the current data did not
388 provide a definitive explanation for the effect of general anesthesia, we previously
389 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 pancuronium bromide did not alter the RBF in cats anesthetized with sevoflurane (data not shown).[26] Finally, we could not investigate to what degree the retinal glial cells contributed to the basal RBF, because it is difficult to quantify the degree of functional damages in the retinal glial cells after intravitreal injection of LAA at a concentration of 60 mM. To resolve this issue, more advanced techniques and research are needed in the future.

Impaired glial cellular activity is related to the pathogenesis of some ocular diseases, such as DR or glaucoma.[9-12] In addition, these ocular diseases also have impaired regulation of ocular blood flow and decreases in flicker-evoked retinal vasodilation.[15,47,49,50] 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. 5). Because it has been reported previously that glial cell dysfunction may be involved in the pathogenesis of DR and glaucoma,[9-12] clarifying the detailed mechanisms of glial cells in the retinal vasculature may provide a further understanding of the pathogenesis in these ocular disorders.

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Legends

FIGURE 1. Effect of LAA on the vasodilatory response to bradykinin. Three groups include five subjects each. The data are expressed as the mean percentage ± standard error of the mean of the pre-injection levels. We used two-way factorial ANOVA followed by the Dunnett procedure to compare LAA (20 and 60 mM) with the control group. $P < 0.05$ is considered statistically significant. There are no significant differences among the three groups. N.S., not significant; D, diameter; V, velocity; F, retinal blood flow.

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 ± standard error of the mean of baseline. $^{*}P < 0.05$ and $^{\dagger}P < 0.05$ compared with a control group by two-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 ± standard error of the mean of baseline. $^{*}P < 0.05$ compared with a control group by one-way factorial ANOVA followed by the Dunnett procedure. D, diameter; V, velocity; F, retinal blood flow.

Figure 3. Effect of L-NPA (nNOS) inhibitor) on the flicker-evoked increase in retinal circulation in LAA-treated eyes. The data are expressed as the mean percentage ± standard error of the mean of baseline. $^{*}P < 0.05$ is considered significant. We used the

two-way factorial ANOVA followed by the Dunnett procedure to compare between before and 2 hours after intravitreal injection of L-NPA in eyes treated with 60 mM of LAA. Flicker-evoked increases in the retinal circulation 2 hours after intravitreal injection of L-NPA (5 mM) in eyes treated with 60 mM of LAA have decreased significantly compared with eyes treated with only 60 mM of LAA.

Figure 4. Photomicrographs of transverse sections of the retina stained with hematoxylin and eosin (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 ganglion cell layer (GCL), inner nuclear cell layer (INL), and outer nuclear layer (ONL) of the retina. Black bar = 50 μm.

Figure 5. 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. White bar = 50 μm.

Figure 6. Scheme of the mechanisms of flicker-induced hyperemia in the retina. Putative mechanisms of the flicker-induced hyperemia in the retina. Three prominent types of vasodilators may contribute to the phenomena.

593 **Tables**

594 **Table 1.** Systemic and Ocular Parameters at Rest

	Control (n=6)	**LAA 20 mM (n=6)**	**LAA 60 mM (n=6)**
pH	7.42 ± 0.01	7.42 ± 0.01	7.43 ± 0.01
$PaCO_2$,mmHg	27.2 ± 1.1	26.1 ± 0.8	26.7 ± 0.2
PaO_2 ,mmHg	140.3 ± 5.9	140.8 ± 4.9	139.6 ± 0.7
HCO_3^-, mmol/l	17.0 ± 0.5	16.8 ± 0.4	16.6 ± 0.1
MABP, mmHg	129.0 ± 2.7	127.1 ± 2.2	128.0 ± 0.7
HR, beats/min	145.0 ± 4.7	149.6 ± 3.9	142.3 ± 3.1
OPP, mmHg	114.0 ± 2.7	112.1 ± 2.2	114.9 ± 2.6
Diameter, μm	114.3 ± 2.2	116.0 ± 2.4	117.0 ± 1.5
Velocity, mm/sec	32.3 ± 2.1	30.2 ± 3.4	29.3 ± 2.4
RBF, μL/min	9.9 ± 0.3	9.6 ± 1.0	9.6 ± 0.9

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596 Control means 0.01 N HCl as a solvent of LAA. Data are expressed as the mean ±
597 standard error of the mean. We used the Mann-Whitney U-test to compare the control
598 group with LAA groups (20 and 60 mM). $P < 0.05$ is considered significant. There are
599 no significant differences between the groups.

600

601 **Table 2.** Systemic and Ocular Parameters Before and 24 Hours after Intravitreal
602 Injection of LAA at 60 mM (n=4)

	Before	After
pH	7.42 ± 0.01	7.40 ± 0.01
$PaCO_2$,mmHg	27.2 ± 1.1	28.6 ± 1.0
PaO_2 ,mmHg	114.3 ± 2.6	111.8 ± 4.1
HCO_3^- ,mmol/l	20.7 ± 0.7	20.1 ± 0.3
MABP, mmHg	103.2 ± 1.0	101.3 ± 1.3
HR, beats/min	121.0 ± 1.4	120.3 ± 4.3
OPP, mmHg	93.6 ± 0.9	91.7 ± 1.4
Diameter, μm	110.6 ± 2.9	110.7 ± 4.0
Velocity, mm/sec	31.1 ± 3.6	34.6 ± 2.9
RBF, μL/min	9.0 ± 1.3	10.2 ± 1.4

603
604 The data are the actual measured values. Each data point (before and after) was
605 measured in the same individual. Data are expressed as the means ± standard error of
606 the mean. To compare before with after injections of LAA (60 mM), we used the
607 Wilcoxon signed-rank test. $P < 0.05$ is considered significant. There are no significant
608 ($P > 0.05$) differences in any parameters between before and after injections. Before
609 indicates before the intravitreal injections; after indicates 24 hours after the intravitreal
610 injections.
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612 **Table 3**. Effect of LAA on ERGs (n=7)

		Control	LAA (60 mM)	P Values
a-wave				
	%Amplitude	103.2 ± 4.6	99.0 ± 5.2	0.38
	%Implicit time	102.5 ± 4.2	99.6 ± 4.4	0.74
b-wave				
	%Amplitude	96.0 ± 5.0	105.1 ± 6.5	0.52
	%Implicit time	100.3 ± 3.1	101.9 ± 3.6	0.45

613

614 Data are expressed as the means ± standard error of the mean of values relative to the
615 baseline for 7 cats each. *P* values obtained using the Mann-Whitney U-test are shown.
616 There are no significant ($P > 0.05$) differences in any parameters between the groups.

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628 **Table 4**. Thicknesses of the GCL,INL, and ONL before and 24 Hours after Intravitreal
629 Injection of LAA (60 mM) (n=5)

	Control	LAA 60 mM	*P* Value
GCL (µm)	16.1 ± 0.8	15.9 ± 0.9	0.59
INL (µm)	17.8 ± 0.5	19.4 ± 0.7	0.16
ONL (µm)	46.6 ± 2.2	50.7 ± 3.0	0.28

630
631 Data are expressed as the mean ± standard error of the mean for 5 cats. *P* values obtained
632 by Mann-Whitney U-test are shown. There are no significant differences ($P > 0.05$) in
633 any layers between groups.

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

	Control	LAA 60 mM	*P* Value
Mean density, pixels/mm	184305.5 ± 26600.2	27675.6 ± 3190.5	0.03

650

651 The mean densities of GFAP expression in astrocytes were compared quantitatively
652 between two groups. Compared with the control, the mean densities of GFAP
653 expression are significantly ($P = 0.03$) reduced in LAA-treated eyes analyzed using the
654 Mann-Whitney U-test. Data are expressed as mean ± standard error of the mean in two
655 groups.

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678 **Supplemental Tables**

679 **Supplemental Table 1.** Effect of BK on Systemic Parameters in Figure 2 (n=5)

	Before	120 min
pH	7.40 ± 0.02	7.41 ± 0.01
$PaCO_2$,mmHg	27.9 ± 1.8	29.6 ± 1.5
PaO_2 ,mmHg	107.4 ± 4.2	110.0 ± 3.8
HCO_3^- ,mmol/l	17.3 ± 0.3	17.5 ± 0.5
MABP, mmHg	114.5 ± 5.0	111.4 ± 4.8
HR, beats/min	133.7 ± 5.1	132.0 ± 3.5
OPP, mmHg	101.5 ± 2.2	99.3 ± 2.6

680

681 Data are expressed as the mean ± standard error of the mean. Before indicates before the
682 intravitreal injections; 120 minutes indicates 120 minutes after the intravitreal injections.
683 The number of animals is 5 in each group. There are no significant (P = 0.05) differences
684 in all parameters between before and 120 minutes after injections.

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695 **Supplemental Table 2.** Effect of L-NPA on systemic Parameters in Figure 3 (n=5)

	Before	120 min
pH	7.41 ± 0.01	7.43 ± 0.01
$PaCO_2$,mmHg	26.5 ± 1.4	26.4 ± 1.7
PaO_2 ,mmHg	108.4 ± 3.1	108.4 ± 3.8
HCO_3^- ,mmol/l	16.5 ± 0.3	16.4 ± 0.4
MABP, mmHg	108.2 ± 3.6	108.6 ± 4.7
HR, beats/min	131.0 ± 4.7	131.0 ± 5.0
OPP, mmHg	98.1 ± 2.6	98.5 ± 2.7

696

697 Data are expressed as the mean ± standard error of the mean. Before indicates before the
698 intravitreal injections; 120 minutes indicates 120 minutes after the intravitreal injections.
699 The number of animals is 5 in each group. There are no significant (P = 0.05) differences
700 in all parameters between before and 120 minutes after injections.

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712 **Figures**

Figure 1.

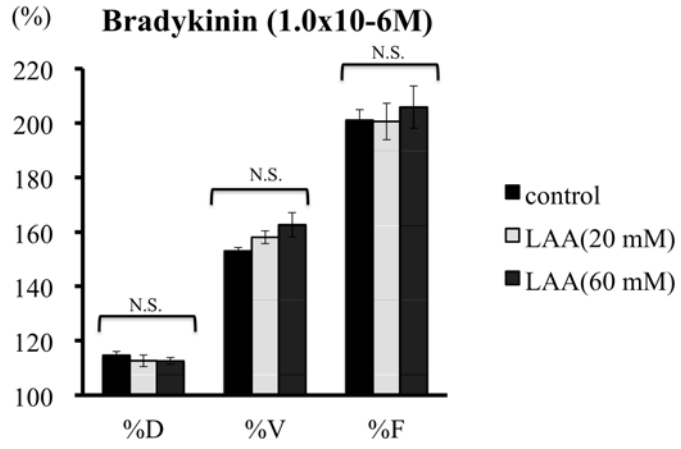

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Figure 2A.

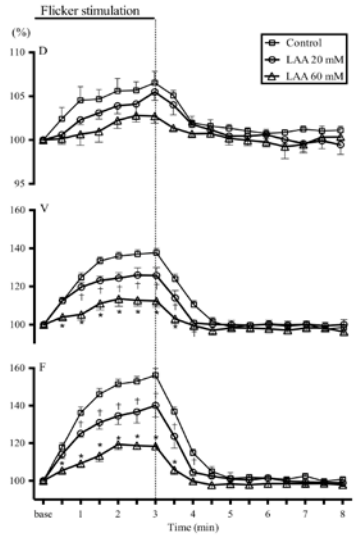

Figure 2B.

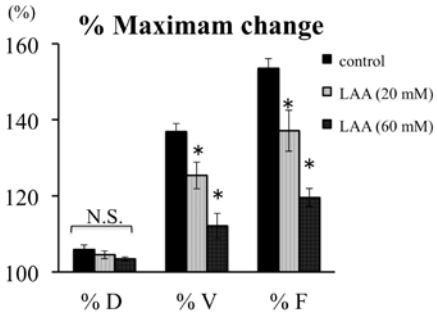

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Figure 3.

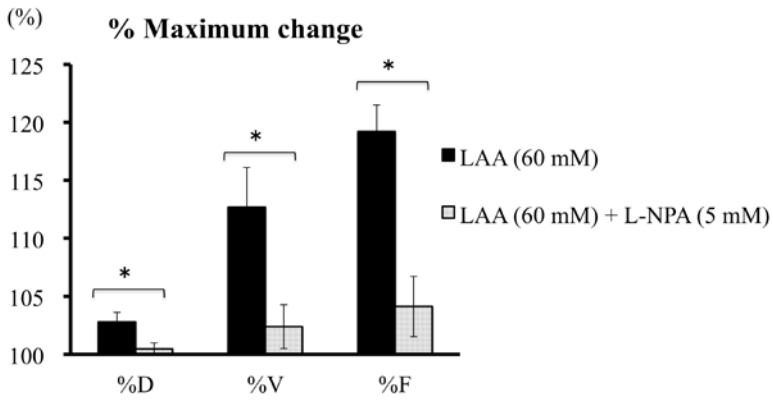

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Figure 4.

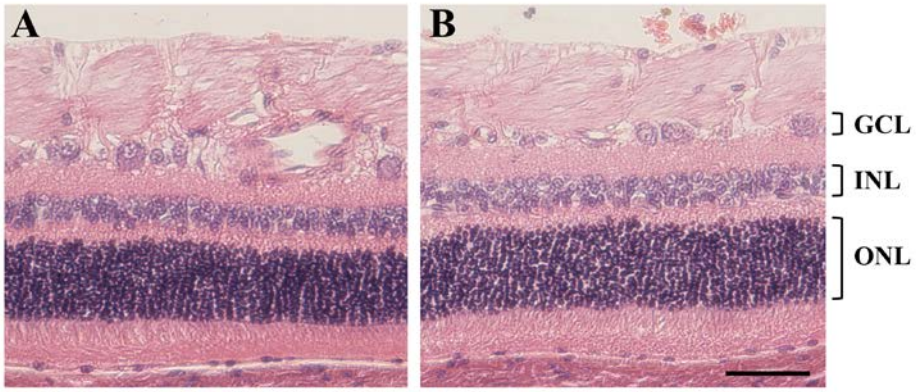

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Figure 5.

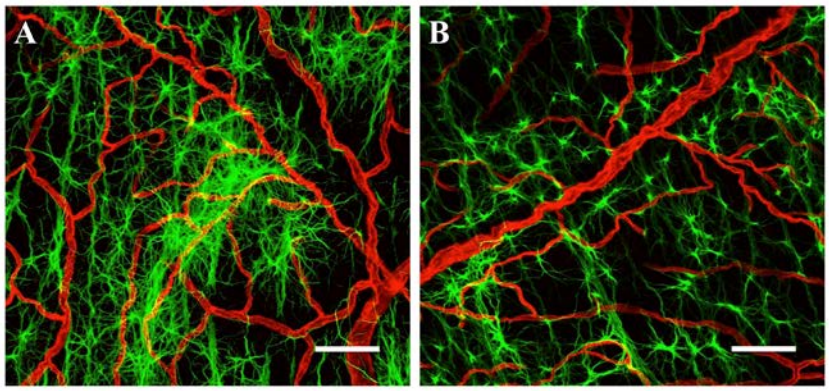

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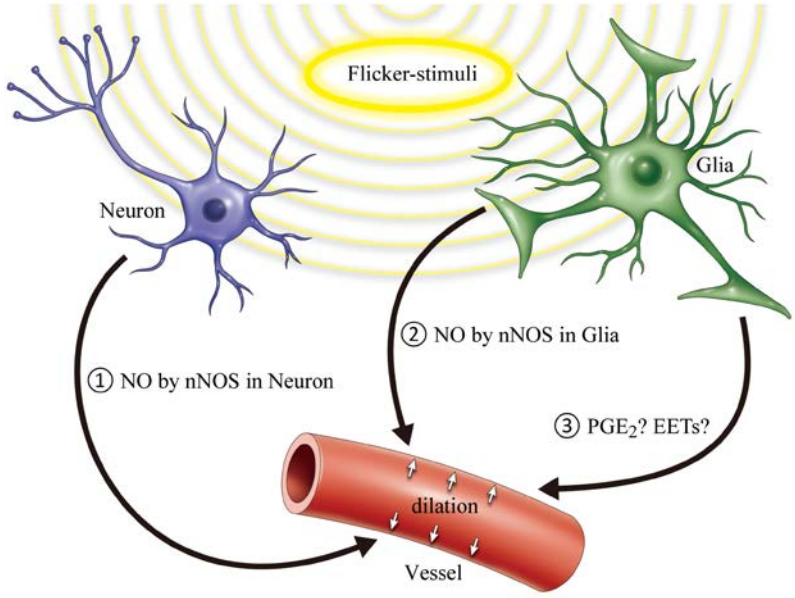
Figure 6.



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