Diffusion Properties of Molecules at the Blood–Brain Interface: Potential Contributions of Astrocyte Endfeet to Diffusion Barrier Functions

Mutsuo Nuriya, Takanori Shinotsuka and Masato Yasui

Department of Pharmacology, School of Medicine, Keio University, Shinjuku, Tokyo 160-8582, Japan

Address correspondence to Mutsuo Nuriya, Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. Email: mnuriya@x2.keio.jp

Molecular diffusion in the extracellular space (ECS) plays a key role in determining tissue physiology and pharmacology. The blood–brain barrier regulates the exchange of substances between the brain and the blood, but the diffusion properties of molecules at this blood–brain interface, particularly around the astrocyte endfeet, are poorly characterized. In this study, we used 2-photon microscopy and acute brain slices of mouse neocortex and directly assessed the diffusion patterns of fluorescent molecules. By observing the diffusion of unconjugated and 10-kDa dextran-conjugated Alexa Fluor 488 from the ECS of the brain parenchyma to the blood vessels, we find various degrees of diffusion barriers at the endfeet: Some allow the invasion of dye inside the endfoot network while others completely block it. Detailed analyses of the time course for dye clearance support the existence of a tight endfoot network capable of acting as a diffusion barrier. Finally, we show that this diffusion pattern collapses under pathological conditions. These data demonstrate the heterogeneous nature of molecular diffusion dynamics around the endfeet and suggest that these structures can serve as the diffusion barrier. Therefore, astrocyte endfeet may add another layer of regulation to the exchange of molecules between blood vessels and brain parenchyma.

Keywords: astrocyte, diffusion, endfoot, extracellular space, 2-photon microscopy

Introduction

The tight regulation of molecular exchange between the brain parenchyma and blood vessels is essential to the maintenance of brain function. This barrier, known as the blood–brain barrier (BBB), is formed from gliovascular structures composed of endothelial cells, astrocytes, and pericytes (Correale and Villa 2009; Abbott et al. 2010). Tight junctions formed by endothelial cells are widely believed to be the cellular substrate of the BBB, while astrocytes and pericytes play important roles in the establishment and maintenance of the BBB (Willis et al. 2004; Armulik et al. 2010; Daneman et al. 2010). However, apart from their supporting roles, the potential functions of astrocyte endfeet in the regulation of molecular diffusion in and out of the gliovascular structure are poorly understood (Nedergaard et al. 2003; Zlokovic 2008). This question has resurfaced with a recent report showing the complete covering of the blood vessel by an intricate and overlapping endfoot (endfoot network), as revealed by detailed 3D reconstructions of electron micrograph images (Mathisien et al. 2010). Indeed, a recent study suggests that the astrocyte endfeet may become a diffusion barrier for water at the blood–brain interface under certain conditions (Haj-Yasein et al. 2011). Since no particularly evident diffusion barrier exists outside of the endfoot network, understanding how molecules diffuse in and out of the brain extracellular space (ECS) through the endfoot network is of particular importance when considering the exchange of biologically active molecules between the brain parenchyma and blood vessels.

So far, most investigations of the barrier functions at the blood–brain interface have been performed by observing the leakage of molecules from inside the blood vessel into the brain parenchyma in vivo. Under these conditions, however, the initial barrier formed by the endothelial tight junctions mask the potential roles of the astrocyte endfeet on diffusion barrier functions. To circumvent this issue, we assessed the diffusion dynamics of molecules from the brain parenchyma to the blood vessel by using an acute brain slice system, a well-established system to study the brain ECS in which the pulsed application of dye to the ECS of the brain parenchyma can be readily performed (Sykova and Nicholson 2008). By combining this system with 2-photon microscopy, which has a good penetration depth and a high spatial resolution, we established a system for assessing the diffusion of fluorescent molecules at and around the astrocyte endfeet. Our analyses reveal highly variable nature of the molecular diffusion dynamics at the endfeet and indicate the contribution of astrocyte endfeet to diffusion barrier functions at the blood–brain interface.

Materials and Methods

Ethical Approval

All the procedures related to the care and treatment of the animals were approved by the Animal Resource Committee of the School of Medicine, Keio University.

Brain-Slice Preparation

C57BL/6j mice (P15–22, SLC, Shizuoka, Japan) were anesthetized with diethyl ether, decapitated, and the brain was quickly removed and placed into an ice-cold cutting solution containing 222 mM sucrose, 27 mM NaHCO3, 2.6 mM KCl, 1.5 mM NaH2PO4, 0.5 mM CaCl2, and 7 mM MgSO4 bubbled with 95% O2/5% CO2 at pH 7.4. Three hundred micrometer-thick acute brain slices were prepared from the visual cortex using a Leica VT1200S Microtome (Leica Microsystems, Wetzlar, Germany). The slices were then transferred to artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 1.14 mM NaH2PO4, 26 mM NaHCO3, 3 mM CaCl2, 1 mM MgSO4, and 10 mM dextrose supplemented with 100–200 nM sulforhodamine 101 (SR101), pre-warmed to 37°C, and bubbled with 95% O2/5% CO2 at pH 7.4; the slices were incubated for 30 min before being cooled to room temperature before use. All the chemicals were purchased from Sigma Aldrich (MO, United States of America), unless otherwise stated.

Imaging

The slices were transferred to a recording chamber perfused with ACSF bubbled with 95% O2/5% CO2 at a speed of 2 mL/min. Glass pipettes (5–20 MΩ; Warner Instruments, CT, United States of America)
were filled with 1 mM unconjugated or 10-kDa dextran-conjugated Alexa Fluor 488 Hydrazide (Invitrogen, CA, United States of America) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) ACSF containing 150 mM NaCl, 3 mM KCl, 10 mM dextrose, and 10 mM HEPES (pH 7.3) and placed 20–50 μm above the surface of the slices. Imaging was performed using an FV1000MPe multiphoton microscopy system (Olympus, Tokyo, Japan) equipped with a Spectra-Physics MaiTai HP femtosecond laser (Newport, CA, United States of America) tuned to 800 nm (∼10 mW under the objective lens) and a LUMPlanFL60×IR2 objective lens (N.A. 0.9, W.D. 2 mm, Olympus). The fluorescence signals of Alexa Fluor 488 and SR101 were simultaneously acquired using 2-channel external photomultiplier tube detectors after an FV10MP-MG/R filter set (BA495-540HQ and BA570-625HQ; Olympus) with ×5 digital zoom at 512 × 512 pixels (0.082 μm/pixel). Time-lapse imaging was performed at room temperature at 0.3 Hz (Kalman averaging of 3 frames taken at 1.1 s/frame, resulting in 3.3 s/frame) for 100 frames in which Alexa Fluor 488 was ejected during frames 1–20. In experiments using 10-kDa dextran-conjugated Alexa Fluor 488, imaging was performed at 0.15 Hz (Kalman averaging of 6 frames taken at 1.1 s/frame, resulting in 6.7 s/frame) to compensate for slow diffusion of this dye compared with unconjugated Alexa Fluor 488. For the oxygen–glucose deprivation (OGD) experiments, the slices were incubated in sucrose-replaced ACSF, in which the glucose had been replaced with sucrose, bubbled with 95% N2/5% CO2 for 75–200 min before analyses. To visualize astrocyte structures (Fig. 1), astrocytes were patch clamped with the internal solution containing Alexa Fluor 488 (200 μM Alexa Fluor 488, 10 mM NaCl, 10 mM KCl, 135 mM KMeSO4, 2.5 mM MgATP, 0.3 mM NaGTP, 10 mM HEPES, pH 7.3) and held under voltage clamp for ∼20 min before imaging.

**Data Analyses**

To analyze the relationship between SR101 and Alexa Fluor 488, the average intensities of 10 images (from frames 11 to 20) were determined and plot profiles of selected regions were obtained using ImageJ (NIH, United States of America). The slopes of the Alexa Fluor 488 signal intensities at the luminal and parenchymal sides were obtained using a linear fit of the Alexa Fluor 488 signal intensities at 7 points (0.49 μm apart) starting from the position at which the peak SR101 signal intensity was recorded (Fig. 4A1), and the analysis was performed using Origin (OriginLab, MA, United States of America). The retention time at each pixel was calculated by fitting the decay phase of the fluorescence intensity changes (frames 21 to 90, Fig. 2B) with a single exponential curve using a custom-written program (MATLAB; Mathworks, MA, United States of America). The slopes of retention time at the endfeet were calculated using a linear fit of the dye retention time data spanning 1.0 μm around the peak of endfeet using Origin (total 13 points, Fig. 5A). The statistical analyses were performed using Origin.

**Results**

**Analyses of Molecular Diffusion at the Blood–Brain Interface**

To visualize the molecular diffusion properties in the brain ECS and to assess the potential roles of the astrocyte endfoot in diffusion barrier functions at the blood–brain interface, we first established a system to visualize molecular diffusion in the vicinity of the astrocyte endfoot. SR101 selectively labels astrocytes when applied to brain tissue (Nimmerjahn et al. 2004; Kafitz et al. 2008). In addition to the cell somata of astrocytes, clearly distinguishable vasculature structures can be observed because of the coverage of the vasculature by the endfoot network (Figs 1 and 2; McCaslin et al. 2011). We confirmed that these SR101-labeled vasculature structures were astrocyte endfeet by visualizing astrocyte structures using intracellular loading with freely diffusible fluorescent molecule, Alexa Fluor 488, through a patch-clamp pipette. This analysis revealed that the SR101-loaded structures at the blood–brain interface were indeed astrocyte endfeet and not other structures such as endothelial cells (Fig. 1).

To investigate the properties of endfeet that were minimally damaged during the preparation of the brain slices, we imaged endfeet at around 100 μm below the surface (near the least-damaged center of the slices). As a probe to characterize the nature of diffusion in the brain ECS, Alexa Fluor 488 (molecular weight, 547; hydrodynamic diameter, 1.1 nm) was used as it does not show any affinity to specific cellular/extracellular structures including transporters expressed at the plasma membrane (Sykova and Nicholson 2008; Xiao et al. 2008; Xiao and Hrabetova 2009). When Alexa Fluor 488 was applied for ∼1 min (20 frames) using pressure ejection through a pipette placed above the brain slices, the dye readily diffused inside the brain slices through the ECS (Fig. 2A,C). Continuous time-lapse imaging of the same field after the end of dye ejection revealed a decay in the fluorescence because of dye diffusion; the decay was well fitted to a single exponential decay curve with the time constant (retention time) when the fluorescence intensity reaches 1/e of the original value (Fig. 2B,C). Using this protocol, maps of the dye-diffusible ECS (Fig. 2C) and the dye retention time (Fig. 2C) at each position could be obtained at the subcellular resolution.

As predicted based on the hydrophilic nature of the Alexa Fluor 488 dye, we found no evidence of dye diffusion or accumulation inside the cells, making the cell somata appear as spots of shadow in the field of view (Kitamura et al. 2008). In addition, the blood vessels also appeared as tubes of shadow, suggesting the existence of an intact BBB that...
excluded the invasion of the Alexa Fluor 488 dye from the ECS of the brain parenchyma into the lumina of the blood vessels (Fig. 3A, B). These data also suggest that these dyes are not actively transported through transporters and, therefore, their distributions reflect the results of passive diffusions in the brain ECS, at least within the timescale of our analyses. When the images of the Alexa Fluor 488 distribution were overlaid with those of endfeet stained with SR101, we found various degrees of the invasion of the Alexa Fluor 488 dye through the endfoot network from the brain parenchyma toward the blood vessels (Fig. 3B). Notably, many of the astrocyte endfeet showed apparent partial or complete blockade of the diffusion, suggesting that astrocyte endfeet may act as a diffusion barrier at the blood–brain interface in addition to endothelial tight junctions (Fig. 3A, B).

**Highly Variable Patterns of Molecular Diffusion at Astrocyte Endfoot Network**

To quantify these results, we calculated the slopes of the Alexa Fluor 488 intensity profiles at the endfeet, both toward (luminal-side slope) and away from (parenchymal-side slope) the center of the endfoot, separately (Fig. 4A). The example shown in Figure 4A1 corresponds to the diffusion pattern at the loose endfeet as shown in Figure 3A1, which results in positive slopes on both luminal and parenchymal sides. All analyses were performed at the z-plane where the distances between the 2 sides of the target endfoot reached a maximum value (Fig. 3). We first checked the stability and reproducibility of this analysis by performing the diffusion analyses repeatedly at the same site. These analyses revealed that the data set from a single vessel fell into the same region during the recording period (<30 min; Fig. 4A, B). Having established the system, data were collected for multiple vessels. These analyses revealed that the invasion of the fluorescent molecules was blocked at astrocyte endfeet in many locations (Fig. 4C: Upper left, 18 of 42 in region I and 6 of 42 in region IIa; overall, 57%). In addition to these populations, we also found a number of cases where Alexa Fluor 488 clearly diffused through the astrocyte endfoot network without any clear diffusion barrier (Fig. 4C, lower right: 3 of 42 in region IIb and 5 of 42 in region III; overall, 19%). The rest of data (10 of 42, 24%) fell into the category IV where the spatial relationship between the endfoot and the diffusion barriers that make accumulation of Alexa Fluor 488 could not be clearly resolved (Fig. 3A3). The width of the endfoot ensheathment around the blood vessel (distances between the 2 sides of the endfeet that are marked with gray dotted lines in Fig. 4A1) used for this analysis ranged from 3.8 to 11 μm (mean, 5.6 μm; standard deviation, 2.0 μm; n = 21). These data suggest 2 key features regarding molecular diffusion in the
vicinity of astrocyte endfeet: 1) The permeability of the astrocyte endfoot network is heterogeneous and 2) the diffusion of molecules from the brain parenchyma to blood vessels is hindered in a large population of endfeet in addition to endothelial cells.

To gain further insights into the molecular diffusion patterns at the blood–brain interface, we also examined the diffusion properties using a bigger probe, 10-kDa dextran-conjugated Alexa Fluor 488 that is similar in size to various bioactive macromolecules such as peptides and proteins (molecular weight, ~10,000; hydrodynamic diameter, 4.5 nm in the case of 10-kDa dextran-conjugated Texas Red; Sykova and Nicholson 2008). Consistent with the data of unconjugated Alexa Fluor 488, this analysis revealed that diffusion of 10-kDa dextran-conjugated Alexa Fluor 488 was also limited outside the endfeet in the large population of gliovascular units (Fig. 4D: Upper left, 18 of 30 in region I and 6 of 30 in region IIa; overall, 80%).

**Astrocyte Endfoot Network Can Create Diffusion-Limited Space**

The data so far suggest that the astrocyte endfeet might restrict the diffusion of molecules between the brain parenchyma and blood vessels. However, the apparent lack of Alexa Fluor 488 dye invasion into the endfoot network can be accounted for by 2 possibilities: 1) The gap between the endothelial cells and the astrocyte endfoot may be so narrow that the amount of dye that can distribute inside the endfoot network is relatively small, despite the presence of loose and permissive connections among the astrocytes or 2) the endfeet form a tight network around the blood vessel, repelling the invasion of dye regardless of the spaces between the astrocytes and endothelial cells and/or pericytes. In the latter case, the dye that manages to pass through the endfoot network would experience a larger diffusion barrier than on the outside, but this difference would not exist or would even be reversed in the former case as in the case where dyes diffuse inside the endfoot network (Fig. 5A). To distinguish between these 2 possibilities, we continuously observed the distributions of Alexa Fluor 488 dye for 70 frames (~4 min) after stopping the dye ejection to measure the dye retention times at each position and to calculate the slopes of retention time at the center of endfoot (Figs 2B, C and 5B). Indeed, this analysis revealed that when dye freely diffuses into the endfoot network (“IN” group; III in Fig. 4A2), the dye inside
the network does not exhibit a longer retention time (Fig. 5C). In contrast, when the blockade of Alexa Fluor 488 dye diffusion through the endfoot network was observed (“OUT” group, I in Fig. 4A2), the dye inside the endfeet clearly persisted longer than the dye outside the network in about half the cases (Fig. 5B, C). A statistical analysis revealed that the slopes of the retention time at the endfeet differed significantly between the IN and OUT groups (Fig. 5C). Together, these data suggest that at least some endfeet form tight connections that are sufficient to block the diffusion of Alexa Fluor 488 dye, consistent with the existence of a tight endfoot network that restricts molecular diffusion (Fig. 5A).

Barrier Functions of Astrocyte Endfeet Collapse Under Pathological Conditions

Finally, we investigated the potential regulation of the permeability of the endfeet. OGD is a well-characterized paradigm that mimics ischemic conditions in brain slices (Strasser and Fischer 1995; Zhao and Flavin 2000; Risher et al. 2009). After extensive exposure to OGD, most of the astrocyte endfoot network became permeable to both unconjugated and 10-kDa dextran-conjugated Alexa Fluor 488 and allowed diffusion inside the network, even at narrow blood vessels with diameters of <4 μm (Fig. 6A, B). When the slopes of Alexa Fluor 488 at the parenchymal side were compared, significant changes in the values that correspond to the permeation of the Alexa dyes were observed after OGD (Fig. 6C). Under these conditions, no signs of Alexa Fluor 488 permeation into the cell soma were observed, suggesting that the membrane integrity of the astrocytes remained intact, consistent with previous reports (Strasser and Fischer 1995; Zhao and Flavin 2000). In addition, dye diffusions into the lumina of blood vessels were still restricted under these conditions (Fig. 6A), indicating that the tight junctions of endothelial cells are still intact, which further support the existence of 2 distinct diffusion barriers formed by astrocytes and endothelial cells. Taken together, these observations suggest that the apparent barrier functions of astrocytes collapse under pathological conditions, making the endfoot permeable to soluble molecules. Furthermore, these observations strongly indicate that the diffusion patterns described above under physiological conditions are not artifacts of the experimental design or analyses, but reflect the actual barrier functions of astrocyte endfeet.

Discussion

In this study, we established a system for evaluating the molecular dynamics in the ECS of the brain slices and applied it to reveal molecular diffusion at the blood–brain interface.

Figure 4. Heterogeneous diffusion properties at astrocyte endfeet. (A) Schematic diagram showing the relationship between the Alexa Fluor 488 diffusion patterns around the astrocyte endfeet and their diffusion slopes. The red and green lines indicate the intensities of the SR101-stained endfeet and the Alexa Fluor 488 signals, respectively. The black arrows indicate the slopes of Alexa Fluor 488 fluorescence intensity profiles at luminal and parenchymal sides, originating from the center of endfoot (gray dotted line; A1). In A2, each section indicates cases where the endfoot block invasion of Alexa Fluor 488 (I), partly (II), or completely (III) allows the invasion of Alexa Fluor 488, or distance between the diffusion boundary and the center of endfoot falls below the spatial resolution of the system (IV). Section II is further divided into 2 cases where the endfoot blockade is strong (IIa) or weak (IIb). Dotted lines in green and red indicate other fluorescence patterns of Alexa Fluor 488 and SR101 than shown in solid lines, which nevertheless result in the same category. (B) Repeatability of the analyses. Data obtained from multiple recordings at the same target site were plotted in the same color. The data were distributed in different regions among the different sites (green, red, and magenta), but were clustered within the same region over multiple recordings. (C) Summary of diffusion patterns of unconjugated Alexa Fluor 488. All the collected data were analyzed and plotted (n = 42). The error bars indicate the standard errors of the linear fits for the calculations of the slopes. (D) Summary of diffusion patterns of 10-kDa dextran-conjugated Alexa Fluor 488. All the collected data were analyzed and plotted (n = 30). The error bars indicate the standard errors of the linear fits for the calculations of the slopes.
**Diffusion Analysis System**

The diffusion properties of ions and molecules in the ECS are key determinants of tissue physiology and pharmacology. To characterize the diffusion properties of molecules in the brain ECS and to investigate the potential function of astrocyte endfeet in the regulation of molecular diffusion, we developed a technique for the direct visualization of molecular dynamics in the brain ECS. To date, diffusion dynamics in the ECS have been mainly studied using 2 methods: The detection of specific ions using ion-selective electrodes and time-lapse imaging of fluorescent molecules released by either iontophoresis or pressure ejection (Sykova and Nicholson 2008). In both cases, however, the analyses are limited by spatial resolution; consequently, the diffusion dynamics at the cellular or subcellular level in the brain ECS have remained a mystery. Two-photon microscopy has the potential to overcome this issue, with its high penetration depth that can reach hundreds of microns below the surface of the tissue with low phototoxicity as well as its high intrinsic spatial resolution in both lateral and axial directions (Stroh et al. 2003). The use of acute brain slices and 2-photon microscopy allowed us to monitor the diffusion of molecules through the brain parenchyma to blood vessels at a subcellular resolution without masking from the diffusion barriers formed by endothelial tight junctions. The stability of the system was confirmed by the repeated measurements at a single site (Fig. 4B); more importantly, the validity of our analyses was strongly supported by the clear changes in diffusion dynamics under pathological conditions (Fig. 6). With its high spatial resolution and non-invasive nature, our system is applicable to a wide variety of targets for studying molecular dynamics under normal/pathological conditions.

Previous studies have revealed that diffusion dynamics of molecules are influenced by the properties of molecules themselves (size, charge, structure, etc.) and the nature of ECS (volume, tortuosity, etc.) (Sykova and Nicholson 2008). While these pioneering studies have provided the key information in considering the diffusion properties of molecules and the nature of brain ECS, these are limited to the macroscopic analyses and there exist little information at the cellular/subcellular level. While the 2-photon microscopy has a potential to overcome this limitation, the subcellular resolution that it has also means that the observations are subject to small disturbances at the target tissue. Therefore, to minimize this possibility, we chose to apply the dye from outside the tissue and measure its diffusion at the center of slices, >100 μm away from an injection site. Although this allowed us to collect data at the blood–brain interface with minimal perturbations, this makes it difficult to compare our results with parameters obtained from similar optical imaging experiments that analyzed diffusion kinetics of probes ejected from a point source placed at the observation point inside the tissue. Furthermore, regional- and species-specific differences may exist in the diffusion properties of molecules at the gliovascular interface. Future studies with different probes under various conditions should help connect the data collected at
these 2 different scales and delineate the diffusion properties of biologically active molecules under physiological/pathophysiological conditions.

Potential Roles of Astrocyte Endfeet on the Diffusion Barrier Functions at the Blood–Brain Interface

Our analyses indicated that 1) diffusion patterns are very heterogeneous around astrocyte endfeet, but 2) some astrocyte endfeet appear to form networks that are tight enough to block the free diffusion of molecules across them, and 3) these barrier functions collapse under pathological conditions. The heterogeneity of the diffusion dynamics can be partly explained by the types of blood vessels and morphological differences in the gliovascular interface, particularly the spaces between the endfeet and endothelial cells (Fig. 5A). With the available information in our system, we could not delineate morphological determinants of molecular dynamics. However, the data regarding the retention time and regulation under pathological conditions strongly suggest that the heterogeneity originates, at least in part, from the nature of the endfoot network itself and that these diffusion patterns observed under the physiological conditions collapse under pathological conditions regardless of the nature of the original heterogeneity (Figs 5 and 6). These findings are consistent with the observation that blood vessels are tightly and completely covered by the endfoot network (Kacem et al.)

Figure 6. Severe tissue damage leads to dye permeation at astrocyte endfeet. (A) Changes in diffusion patterns upon exposure to OGD conditions. Images of Alexa Fluor 488 and SR101 in a brain slice at 0 (under normal conditions) and 75 min (after exposure to OGD conditions) are shown (left). Intensity profiles (right) of the same area (middle panels) reveal the changes in the diffusion patterns. (B) Summary graph of Alexa Fluor 488 invasion under OGD conditions. The slopes of unconjugated (B1, n = 16) and 10-kDa dextran-conjugated (B2, n = 28) Alexa Fluor 488 diffusion were calculated and plotted. (C) Comparison of parenchymal side slopes among groups. The slopes of unconjugated (C1) and 10-kDa dextran-conjugated (C2) Alexa Fluor 488 at the parenchymal side of the endfeet were plotted. The open circles show the individual data and the filled squares indicate the mean values of the data with whiskers showing the standard deviations in each group. The OGD groups have significantly smaller parenchymal slope values than the control groups for both unconjugated Alexa Fluor 488 (C1, P < 1 × 10−3, Mann–Whitney test; normal, n = 32; OGD, n = 10) and 10-kDa dextran-conjugated Alexa Fluor 488 (C2, P < 5 × 10−3, Mann–Whitney test; normal, n = 26; OGD, n = 13).
result in reduction in the ECS between overlapping endfeet, and Norenberg 2005). Such swelling in astrocyte endfeet may be the earliest and most prominent changes in this process (Panickar et al. 2003). While ischemic conditions are known to induce a wide variety of changes in gliovascular structures, severe tissue damage caused by OGD resulted in this possibility by employing the OGD paradigm. Under our condition, severe tissue damage caused by OGD resulted in disruption of apparent diffusion barriers around the astrocyte endfoot, whereas those at the endothelial tight junctions remained intact. While ischemic conditions are known to induce a wide variety of changes in gliovascular structures, swelling of astrocyte endfoot is considered to be one of the earliest and most prominent changes in this process (Panickar and Norenberg 2005). Such swelling in astrocyte endfeet may result in reduction in the ECS between overlapping endfeet, which may lead to even more limited diffusion of molecules across endfoot ensheathments. At least under our experimental condition, however, we did not observe such effect and found that endfoot ensheathment actually became permeable to molecules under OGD. Previous studies have shown that the tight relation between endfeet and basal lamina collapses under ischemic condition (Wang and Shuaib 2007; Kwon et al. 2009). Together with the importance of basal lamina for astrocyte physiology, we speculate that OGD leads to the disruption of intricate and organized structures of the endfoot network supported by basal lamina that constitutes diffusion-limited spaces under normal conditions. While mechanistic understandings of the regulation of diffusion barriers at the astrocyte endfoot need further investigations, our data underscore the importance of functional analyses supplementing structural information in elucidating the dynamics of molecules in the brain ECS.

### Limitations

While our analysis system has key advantages over previously techniques, several limitations warrant attentions. First and foremost, potential changes in the diffusion properties in the in vitro system need to be kept in mind. While brain slices allow us to readily apply probes to the ECS without further perturbation, lack of circulation and tissue damages at the surface of the tissue may affect some properties at the blood–brain interface. In this regard, the fact that the blood vessels are open-ended and are connected to subpial space could potentially affect our analyses on diffusion properties at the blood–brain interface. Although our choice of relatively small-diameter blood vessels located far from the surface as well as pulse application of the dye in a short period minimize the effects of dyes coming from these open ends, contributions of these dyes might have led us to underestimate diffusion barrier functions of astrocyte endfeet. Limitation also arises from the spatial resolution of our system (~500 nm), which is not high enough to determine the thickness of individual endfoot around blood vessels (<500 nm; Mathiisen et al. 2010). While it was still possible to identify the center positions of the endfoot, further morphological analyses addressing the potential relationship between the thickness of the endfeet and their diffusion barrier functions could not be performed due to this limitation. Finally, what we characterized in this study was the molecular diffusion from the brain parenchyma into the lumen of blood vessels. As such, while we expect that the same diffusion patterns apply regardless of the directions for molecules following the passive and random diffusions, we cannot exclude a possibility that direction-specific diffusion patterns exist, particularly when active mechanisms are involved.

### Potential Regulation of Molecular Diffusion at the Blood–Brain Interface

Accumulating evidence suggest that the morphology of astrocytes are dynamically regulated under physiological/pathophysiological conditions (Haber et al. 2006; Nishida and Okabe 2007; Risher et al. 2009). Considering this dynamic nature of astrocyte, it is tempting to speculate that astrocyte endfeet may function as a dynamically regulated barrier for molecular exchange at the blood–brain interface. We tested this possibility by employing the OGD paradigm. Under our condition, severe tissue damage caused by OGD resulted in disruption of apparent diffusion barriers around the astrocyte endfoot, whereas those at the endothelial tight junctions remained intact. While ischemic conditions are known to induce a wide variety of changes in gliovascular structures, swelling of astrocyte endfoot is considered to be one of the earliest and most prominent changes in this process (Panickar and Norenberg 2005). Such swelling in astrocyte endfeet may result in reduction in the ECS between overlapping endfeet.

### Funding

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (KAKENHI #20790211, 23657106 to M.N.), Global COE Program “Center for Human Metabolomic Systems Biology” from MEXT, Japan (M.Y.), Keio University Grant-in-Aid for Encouragement of Young Medical Scientists (M.N.), and the Mochida Foundation (M.N.).
Notes
We thank members of the laboratory for comments and Olympus cooperation for technical supports. Conflict of Interest: None declared.

References