Induction of xCT Gene Expression and l-Cystine Transport Activity by Diethyl Maleate at the Inner Blood–Retinal Barrier

Masatoshi Tomi,1,2,3 Ken-ichi Hosoya,1,2 Hitomi Takanaga,2,3,4 Sumio Obitsu,2,3,4 and Tetsuya Terasaki2,3,4

Purpose. In this study, the expression and regulation of the l-cystine transporter, system xCT−, at the inner blood-retinal barrier (inner BRB) was investigated using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2) as an in vitro model.

Methods. For the uptake study, TR-iBRB2 cells were cultured at 33°C in the presence or absence of diethyl maleate (DEM), and the uptake rate of [14C]l-cystine was measured at 37°C. The mRNA levels of system xCT, which consists of xCT and 4F2hc, were determined by quantitative real-time RT-PCR analysis with specific primers.

Results. The xCT and 4F2hc mRNAs were expressed in TR-iBRB2 cells. The [14C]l-cystine uptake by TR-iBRB2 cells appeared to be mediated through a saturable Na+-dependent process. The corresponding Michaelis-Menten constant was 9.18 μM. At 100 μM DEM, the xCT mRNA level and l-cystine uptake activity in TR-iBRB2 cells were enhanced in a time-dependent manner. Concomitantly, the glutathione concentration in TR-iBRB2 cells was increased. In contrast, the 4F2hc mRNA level was unchanged up to 24 hours and was induced for more than 24 hours by DEM treatment. Under both normal and DEM treatment conditions, the uptake of [14C]l-cystine was strongly inhibited by l-glutamic acid, l-α-aminoacipic acid, l-homocysteic acid, and l-quissualic acid, whereas l-aspartic acid and l-arginine had no effect, which is evidence of the induction of system xCT−.

Conclusions. System xCT−-mediated l-cystine uptake appears to be present at the inner BRB. DEM induces l-cystine transport through system xCT− at the inner BRB by enhanced transcription of the xCT gene. (Invest Ophthalmol Vis Sci. 2002;43:774–779)

The retina is unique among body tissues, because it is the only tissue in which light is focused on a group of cells. It is necessary to protect the retina against oxidative stress, because light causes free radical oxidation.1 Glutathione plays a key role in protecting cells against free radicals, peroxides, and other toxic compounds, and it is also important for protecting against the harmful effects of exposure to an oxidizing environment.2 Glutathione also modulates synaptic transmission in the retina.3 L-Cysteine (L-Cys) is one of the rate-limiting precursors of glutathione synthesis.4 However, the concentration of l-Cys in the plasma (10–20 μM) is 10 times lower than that of l-Cystine (100–200 μM), because it is present as a dimer in plasma.5 After l-cystine undergoes uptake into cells, it is rapidly reduced to l-Cys.6 Under conditions of oxidative stress in the retina, therefore, it is necessary that it undergoes influx transport from the circulating blood to the retina across the blood–retinal barrier (BRB) to synthesize glutathione to protect the retina.

L-Cysteine and l-glutamic acid (l-Glu) exchange transporter, referred to as system xCT−, is composed of the heavy chain of 4F2 cell-surface antigen (4F2hc/CD98) and xCT protein.7–9 The physiological flux through system xCT− involves the entry of l-cystine and the exit of l-Glu. In addition, system xCT− is induced after an 8-hour culture with 100 μM diethyl maleate (DEM) and/or 1 ng/mL lipopolysaccharide.10 DEM is often used as a reagent to deplete intracellular glutathione to induce oxidative stress, because it is relatively less toxic than some other electrophilic agents.7,9,10 By means of in vivo integration plot analysis, we recently showed that l-cystine uptake in eye and brain is activated after a 12-hour DEM infusion from the external carotid artery, and this enhanced uptake is inhibited in the presence of l-Glu and l-α-aminoacidic acid (l-AAA), substrates for system xCT−. This suggests that l-cystine influx transport through system xCT− is activated by DEM at the blood-brain barrier (BBB) and BRB in vivo.11

The BRB, which is composed of retinal capillary endothelial cells (inner BRB) and retinal pigmented epithelial cells (outer BRB), may play a key role in influx and efflux transport from the circulating blood to the retina.12 Very recently, Bridges et al.13 reported that xCT and 4F2hc were expressed in a cultured human retinal pigment epithelial cell line, and xCT mRNA was induced by a nitric oxide donor, 3-nitroso-N-acetylenicillamine. This induction appeared to act as an antioxidant protection mechanism.14 The glutathione transporter (RcGshT) is also expressed in cultured human retinal pigment epithelial cells.15 However, our knowledge of the l-cystine transport mechanism and regulation of l-cystine transporter at the inner BRB is still incomplete. It is important to have more information about the l-cystine transport system at the inner BRB under normal and oxidative stress conditions, because the inner two thirds of the human retina is nourished by a direct blood supply through the inner BRB.14

We recently established conditionally immortalized rat retinal capillary endothelial cell lines (TR-iBRB) from a transgenic rat harboring temperature-sensitive simian virus (SV)40 large

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T-antigen gene. TR-iBRB cells possess endothelial markers and express D-glucose transporter (GLUT1), efflux transporter (P-glycoprotein), and monocarboxylate transporter-1 (MCT1), which have been reported to be involved in the expression at the inner BRB, detected by immunohistochemical analysis. Thus, TR-iBRB cells maintain certain in vivo transport functions and are suitable in vitro model for the inner BRB.

The purpose of the present study was to investigate the cystine transport mechanism and the expression and regulation of system x_c^- under normal and oxidative stress conditions, using TR-iBRB cells as an in vitro model of the inner BRB.

**Materials and Methods**

**Animals**

Male Wistar rats, weighing 250 to 300 g, were purchased from Charles River (Yokohama, Japan). The investigations involving animals conformed to the provisions of the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Cell Culture**

TR-iBRB2 cells were established and characterized as described previously. TR-iBRB2 cells were seeded onto rat tail collagen type I-coated tissue culture dishes (BD Biosciences, Bedford, MA). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Moregate, Bulimbra, Australia) and 15 μg/L endothelial cell growth factor (Roche Molecular Biochemicals, Mannheim, Germany) in the presence or absence of DEM (Wako Pure Chemicals, Osaka, Japan), which is a sulfhydryl-reactive agent, at 33°C, due to the presence of temperature-sensitive SV40 T-antigen gene. TR-iBRB cells possess endothelial markers and express D-glucose transporter (GLUT1), efflux transporter (P-glycoprotein), and monocarboxylate transporter-1 (MCT1), which have been reported to be involved in the expression of system x_c^- under normal and oxidative stress conditions, using TR-iBRB2 cells as an in vitro model of the inner BRB.

**Reverse Transcription-Polymerase Chain Reaction Analysis**

Total cellular RNA was prepared from phosphate-buffered saline (PBS)-washed cells using Trizol reagent (Gibco BRL, Rockville, MD). Single-strand cDNA was made from 1 μg total RNA by reverse transcription (RT) using oligo dT primer. The polymerase chain reaction (PCR) was performed using a gene amplification system (GeneAmp PCR system 9700; PE-Applied Biosystems, Foster City, CA) with xCT- or 4F2hc-specific primers through 40 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute (Table 1). The PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide and visualized using an imager (Epiprio 7000; Aisin, Aichi, Japan). The PCR products of the expected length were cloned into a plasmid vector (pGEM-T Easy Vector System I; Promega, Madison, WI) and amplified in Escherichia coli. Several clones were sequenced from both directions using a DNA sequencer (model 4200; LI-COR, Lincoln, NE).

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using a sequence detector system (Prism 7700; PE-Applied Biosystems) with a kit (SYBR Green PCR Master Mix; PE-Applied Biosystems), according to the manufacturer’s protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using the plasmid (pGEM-T Easy Vector; Promega) containing the gene of interest. This enabled standardization of the initial mRNA content of TR-iBRB2 cells relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR was performed using xCT-, 4F2hc-, GLUT1-, or GAPDH-specific primers (Table 1) and the cycling parameters stated earlier.

**Determination of Intracellular Glutathione**

Measurement of the total glutathione of PBS-washed cells using a kit (Biosyntech GSH-420, Oxis Research International, Portland, OR) was performed according to the manufacturer’s protocol. The method is based on the formation of a chromophoric thione. Protein assay was performed with a kit (DC; Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard.

**Data Analysis**

For kinetic studies, the Michaelis-Menten constant (K_m), maximum uptake rate (V_max), and nonsaturable uptake rate constant (P_nonsat) of l-cystine uptake were calculated from equation 1, using a nonlinear least-squares regression analysis on computer.  

\[
V = V_{max} \times C/(K_m + C) + P_{nonsat} \times C
\]  

(1)

where V and C are the uptake rate of l-cystine at 5 minutes and the concentration of l-cystine, respectively. To analyze the mechanism for the inhibition by l-Glu (300 μM) the inhibitory constant (K_i) was calculated from equation 2 on computer.  

\[
V = V_{max} \times C/(K_m \times (1 + I/K_i) + C)
\]

(2)

where I is the concentration of l-Glu.

**Table 1. Oligonucleotide Primers Used for PCR Amplification of cDNAs**

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Upstream Primer (5’ to 3’)</th>
<th>Downstream Primer (5’ to 3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xCT</td>
<td>CCTGGCAATTGAGGCTACAT</td>
<td>TCAGAATTGCTGTAGGCTGA</td>
<td>182</td>
</tr>
<tr>
<td>4F2hc</td>
<td>CTCCGAAGAGATTATAAGACCTTCT</td>
<td>TCAGTATTGGCTGCTACATAGTCAG</td>
<td>141</td>
</tr>
<tr>
<td>GLUT1</td>
<td>GAGTGAACCTGTTTGCTCCATG</td>
<td>AGGGGACAGCTCTCGAGATG</td>
<td>503</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGATGACATCGAGAGGTGGTGAAG</td>
<td>TCTTTAGGAGGCGATGAGGCCAT</td>
<td>240</td>
</tr>
</tbody>
</table>

**References**

1. ACI. 2002, Vol. 43, No. 3.
protein at 10 and 30 minutes, respectively. This supports an enzyme for TR-iBRB2 cells.

Lane 1: rat brain as a positive control in both xCT (A) and 4F2hc (B); lane 2: TR-iBRB2 cells; lane *: in the absence of reverse transcriptase for TR-iBRB2 cells.

Unless otherwise indicated, all data represent the mean ± SEM. Statistical significance of differences among means of several groups was determined by one-way analysis of variance (ANOVA) followed by a modified Fisher least-squares difference method.

RESULTS

Expression of xCT and 4F2hc mRNA in TR-iBRB2 cells

The expression of xCT and 4F2hc mRNA in TR-iBRB2 cells was analyzed by RT-PCR. The bands corresponding to the expected 182- and 141-bp for xCT and 4F2hc, respectively, were amplified from TR-iBRB2 cells, with rat brain as a positive control (Fig. 1A, 1B).7,9,23 The DNA sequence of the bands of TR-iBRB2 cells was almost identical with that of mouse7 and human8,9 (100%).

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**1-Cystine Uptake by TR-iBRB2 Cells through System xC**

The time course of [14C]-cystine uptake by TR-iBRB2 cells is shown in Figure 2. [14C]-Cystine uptake increased linearly for at least 30 minutes. The [14C]-cystine uptake (cell-to-medium ratio) was 89.8 ± 5.1 μL/mg protein and 240 ± 6 μL/mg protein at 10 and 30 minutes, respectively. This supports an apparently concentrative behavior, because the [14C]-cystine uptake was larger than the cell volume of approximately 3 μL/mg protein, estimated by 3-O-methyl-D-glucose uptake through facilitated D-glucose transporter, GLUT1, in TR-iBRB2 cells.15

**Figure 3** shows the concentration-dependent uptake of L-cystine by TR-iBRB2 cells. In an analysis using an Eadie-Scatchard plot, the intracellular L-cystine uptake was found to consist of saturable and nonsaturable processes (Fig. 3, inset). Nonlinear least-squares regression analysis revealed that the maximum initial uptake rate was 89.8 μmol/(min·mg protein), and the Michaelis constant was 75.4 ± 10.8 μmol/(min·mg protein) (mean ± SD).

**Figure 3**. Concentration-dependent L-cystine uptake by TR-iBRB2 cells. The [14C]-cystine (1.7 μM) uptake was performed at 5 minutes and 37°C. Each point represents the mean ± SEM (n = 4). Data were subjected to Michaelis-Menten and Eadie-Scatchard analyses (inset). The Kₘ, Vₘₕ, and Pₘₙ were 9.18 ± 3.18 μM, 75.4 ± 10.8 μmol/(min·mg protein), and 0.410 ± 0.029 μL/(min·mg protein) (mean ± SD), respectively.

The inhibitory effect of Na⁺-free conditions on [14C]-cystine uptake by TR-iBRB2 cells was examined under two different sets of conditions. The choline ECF buffer reduced slightly the [14C]-cystine uptake by 26.2% ± 2.7% (P > 0.05). Interaction between the organic cation, choline, and a tracer level of cystine uptake by TR-iBRB2 cells takes place in an Na⁺-independent manner.

The inhibition study was performed to characterize the [14C]-cystine uptake by TR-iBRB2 cells under normal conditions (Table 2, No pretreatment). [14C]-Cystine uptake was inhibited by more than 80% by l-cystine, l-Glu, l-AAA, l-homocysteic acid, l-homocysteic acid, and l-homocysteic acid, respectively.

**Table 2. Effect of Several Inhibitors on [14C]-Cystine Uptake by TR-iBRB2 Cells**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>No Pretreatment</th>
<th>DEM Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>100 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>DEM pretreatment</td>
<td>100 ± 4</td>
<td>—</td>
</tr>
<tr>
<td>1 mM l-Cystine</td>
<td>3.19 ± 0.23*</td>
<td>3.20 ± 0.04*</td>
</tr>
<tr>
<td>1 mM l-Cystine</td>
<td>51.9 ± 2.8*</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM L-Glutamic acid</td>
<td>9.67 ± 0.52*</td>
<td>19.3 ± 0.6*</td>
</tr>
<tr>
<td>2.5 mM D-Glutamic acid</td>
<td>73.0 ± 3.2†</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM l-O-Aminoadipic acid</td>
<td>10.9 ± 0.6*</td>
<td>19.3 ± 0.6*</td>
</tr>
<tr>
<td>2.5 mM L-Homocteic acid</td>
<td>7.62 ± 0.83*</td>
<td>6.12 ± 0.15*</td>
</tr>
<tr>
<td>2.5 mM L-Quisqualic acid</td>
<td>19.2 ± 0.6*</td>
<td>13.5 ± 0.4*</td>
</tr>
<tr>
<td>2.5 mM L-Aspartic acid</td>
<td>89.7 ± 2.8</td>
<td>86.7 ± 3.5</td>
</tr>
<tr>
<td>2.5 mM L-Leucine</td>
<td>83.0 ± 7.2</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM L-Arginine</td>
<td>104 ± 7</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>2.5 mM L-Lysine</td>
<td>76.5 ± 4.2‡</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM γ-Aminobutyric acid</td>
<td>94.0 ± 2.4</td>
<td>ND</td>
</tr>
<tr>
<td>2.5 mM p-Aminohippuric acid</td>
<td>99.7 ± 1.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

[14C]-Cystine (1.7 μM) uptake was performed for 10 minutes. [14C]-Cystine uptake in DEM pretreatment control was 2.2-fold greater than that in no-pretreatment control. Data are mean percentage of control ± SEM (n = 4–12). ND, not determined.

* P < 0.001; † P < 0.05; ‡ P < 0.01, significantly different from the respective control.
cysteic acid (L-HCA), and D-quiralic acid (D-QQA), all of which are substrates for system xc. It was partly inhibited by L-glutamine (L-Gln), L-lysine (L-Lys) by up to 48%, whereas L-aspartic acid (L-Asp), L-leucine (L-Leu), L-arginine (L-Arg), γ-aminobutyric acid (GABA), and D-aminohippuric acid (PAH) had no effect on [14C]L-cystine uptake. Moreover, the Lineweaver-Burk plot showed that the two lines of the L-cystine uptake intersected on the ordinate. This indicates that L-Glu competitively inhibited L-cystine uptake with a Ki of 142 ± 18 μM (mean ± SD; Fig. 4).

**Effect of DEM in TR-iBRB2 Cells**

The effects of DEM treatment on mRNA expression, [14C]L-cystine uptake, and glutathione concentration were examined in TR-iBRB2 cells. Treatment with 100 μM DEM resulted in time-dependent induction of xCT mRNA up to 48 hours, whereas GLUT1, used as an unrelated transporter, did not change (Fig. 5A), supporting the hypothesis that xCT mRNA is specifically induced by DEM treatment. Corresponding to xCT mRNA expression, the [14C]L-cystine uptake was enhanced up to 24 hours in a time-dependent manner and reached a plateau at more than 24 hours (Fig. 5B). Moreover, the intracellular glutathione concentration was also increased in a time-dependent manner (Fig. 5B). The xCT mRNA level, [14C]L-cystine uptake activity, and the glutathione concentration after 24 hours of 100 μM DEM treatment were 2.61-, 2.33-, and 1.43-fold greater than that of the control (at time 0), respectively. In contrast, the expression of 4F2hc mRNA was unchanged up to 24 hours and induced at 36 and 48 hours (Fig. 5A).

Figure 6 shows the effect of DEM concentration (24-hour treatment) on the mRNA expression, [14C]L-cystine uptake, and glutathione concentration in TR-iBRB2 cells. The expression of xCT mRNA was significantly induced at DEM concentrations of 100 to 300 μM, and the maximum induction took place at 200 μM (Fig. 6A), whereas the [14C]L-cystine uptake was enhanced in a dose-dependent manner. At 100 to 200 μM DEM, the intracellular glutathione concentration was significantly increased, with maximum glutathione concentration occurring at 100 μM DEM (Fig. 6B). In contrast, the expression of 4F2hc and GLUT1 mRNA was not significantly affected in the presence of 50 to 500 μM DEM (Fig. 6A).

An inhibition study was performed to characterize the [14C]L-cystine uptake by TR-iBRB2 cells after treatment with 100 μM DEM for 24 hours (Table 2, DEM pretreatment). [14C]L-Cystine uptake was inhibited by more than 80% by L-cystine, L-Glu, L-AAA, L-HCA, and L-QQA, whereas L-Asp and L-Arg produced no inhibition. The manner of inhibition was identical with that of [14C]L-cystine uptake under normal conditions.

**DISCUSSION**

In the present study, TR-iBRB2 cells used as an in vitro model of the inner BRB expressed xCT and 4F2hc mRNA (Fig. 1), and L-cystine uptake occurred in an Na+-independent and concentration-dependent manner (Fig. 3). The corresponding Km of 9.18 μM is 8.8-fold lower than that obtained for L-cystine uptake (Km = 81 μM), using mouse xCT and 4F2hc cRNA coinjected Xenopus laevis oocytes. There was no agreement of Km probably because of a difference between species and/or the experimental systems. Nevertheless, [14C]L-cystine uptake was strongly inhibited by system xc substrates, such as L-Glu, L-AAA, L-HCA, and L-QQA (Table 2). This manner of inhibition is consistent with system xc characteristics, as reported elsewhere. System bo, which is also an Na+-independent transporter, mediates the transport of L-cystine, L-Leu, and basic amino acids. System bo-mediated L-cystine uptake by TR-iBRB2 cells excludes the involvement of system bo, because L-Leu, L-Arg, and L-Lys produced no marked inhibition (Table 2). Moreover, [14C]L-cystine uptake was competitively inhibited by L-Glu with a Ki of 142 μM (Fig. 4), which is very close to the Km of L-Glu uptake (200 μM) by cultured human fibroblasts.
This evidence supports the finding that activation of L-cystine uptake through system x_{c}^{-} in TR-iBRB2 cells stimulates glutathione synthesis. However, DEM at more than 300 μM depleted intracellular glutathione (Fig. 6B) and injured cells, because the protein content per dish was reduced by 33% and 75% at 300 and 500 μM DEM compared with the control, respectively (data not shown). This is in good agreement with a previous result in human fibroblasts and suggests that DEM acts in two different ways: as an inducer of xCT mRNA at 100 μM DEM and as a nonspecific deleterious agent at higher concentrations. Therefore, the xCT level declined at more than 300 μM DEM. However, it is not clear at present whether L-cystine uptake was increased up to 500 μM DEM. Further studies are needed to investigate the protein level of xCT expression.

In contrast, the 4F2hc mRNA level did not change up to 24 hours at a DEM concentration of 0 to 500 μM (Figs. 5A, 6A), as was the case with the human retinal pigment epithelial cell line, probably because the amount of 4F2hc mRNA was 56-fold greater than xCT mRNA, according to quantitative real-time PCR analysis under normal conditions (data not shown). Therefore, 4F2hc protein was large enough to bind to xCT protein, even though xCT mRNA was increased by 2.61-fold during the 24-hour DEM treatment. Moreover, 4F2hc protein is a component of several other amino acid transport systems, such as systems L and y^{+}L. However, the 4F2hc mRNA was increased for longer than the 24-hour DEM treatment (Fig. 5A). One possibility is that other amino acid transporters are induced for more than 24 hours. The inner BRB may express system L because of the uptake of large neutral amino acids, as occurs in isolated bovine retinal capillary. Taking all these results into consideration, we conclude that system x_{c}^{-} is expressed in TR-iBRB2 cells and the induction of xCT, activation of L-cystine uptake, and enhancement of glutathione synthesis occur under 100-μM DEM treatment for 24 hours.

A number of possible physiological roles for the induction of system x_{c}^{-} include action as a detoxifying system in the retina and retinal capillary endothelial cells by supplying L-cystine/L-Cys for the synthesis of glutathione. Hyperglycemia is associated with an increased production of reactive oxygen species and accumulation of oxidative damage in various tissues. Moreover, oxidative damage at the inner BRB and in the retina is thought to be involved in retinal diseases, such as diabetic retinopathy and age-related macular degeneration. In the retinal Müller cells, γ-glutamylcysteine synthetase subunit gene expression is induced under oxidative stress conditions, suggesting that glutathione synthesis is enhanced under such conditions in the retina, and L-Cys is also required in the retina from the circulating blood for glutathione synthesis. This evidence from our current in vitro study and previous in vivo results suggests that L-cystine undergoes influx transport from the circulating blood to the retina across the inner BRB under oxidative stress conditions after DEM treatment to protect the retina from oxidative damage. However, there remains the possibility that synthesized glutathione in retinal capillaries undergoes efflux to the retinal parenchymal cells. Müller cells are known to surround the retinal capillary, and glutathione in the retina is mainly produced in Müller cells.

In conclusion, the xCT mRNA level, L-cystine transport activity, and glutathione levels were enhanced under oxidative stress conditions after DEM treatment of TR-iBRB2 cells used as an in vitro model for the inner BRB, and L-cystine uptake into the eye was enhanced after a 12-hour DEM infusion in vivo. These findings are an important contribution to a better understanding of the supply of L-cystine to the retina as well as to the retinal capillaries and of the detoxifying role of the inner BRB.
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References
