**Regulatory Article**

**In Vitro** Study of L-Glutamate and L-Glutamine Transport in Retinal Pericytes: Involvement of Excitatory Amino Acid Transporter 1 and Alanine–Serine–Cysteine Transporter 2

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L-Glutamate (L-Glu) is known to be a relaxant of pericytes and to induce changes in microcirculatory hemodynamics. Since the concentration of L-Glu which induces the dilation of retinal capillaries is reported to be high compared with the estimated concentration in the retinal interstitial fluid, it is hypothesized that some systems involving concentrative L-Glu release are present in retinal pericytes. The purpose of this study was to investigate the existence of L-Glu-storing systems, which contribute to autocrine L-Glu release, in retinal pericytes using conditionally immortalized rat retinal pericytes (TR-rPCT1 cells), which express mRNAs of L-Glu-synthesizing enzymes from L-glutamine (L-Gln). TR-rPCT1 cells express the mRNAs of vesicular L-Glu transporter 1 (VGLUT1), indicating that L-Glu in the cytoplasm is taken up into VGLUT1-expressing vesicles of retinal pericytes. L-Glu and L-Gln are taken up into TR-rPCT1 cells via Na\(^+\)-dependent saturable processes with a \(K_m\) value of 22.4 \(\mu\)M and 163 \(\mu\)M, respectively. The \(^{3}H\)L-Glu uptake was inhibited by ca. 50% in the presence of \(\alpha\)-aspartate, a substrate of excitatory amino acid transporter (EAAT) subtypes, whereas substrates of alanine–serine–cysteine transporter (ASCT) subtypes exhibited only a weak inhibitory effect on the uptake. The inhibitory effect of ASCT substrates on the \(^{3}H\)L-Glu uptake was stronger than that of other neutral amino acid transport systems. Consequently, it was determined that EAAT1 and ASCT2 play a role in the transport of L-Glu and L-Gln, respectively, from retinal interstitial fluid to the cytoplasm of retinal pericytes.

**Key words** excitatory amino acid transporter; L-glutamate; retinal pericyte; alanine–serine–cysteine transporter; L-glutamine

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Retinal pericytes reside within the basal lamina of retinal capillaries and communicate with the retinal capillary endothelial cells which form the blood–retinal barrier (BRB). It is known that the contraction and relaxation of pericytes which are induced by physiological factors leads to a change in the diameter of the capillaries. Because it has been reported that the diameter of capillaries in the central nervous system (CNS) affects the blood flow rate in the capillaries and, thus, the exchange of some compounds between the circulating blood and retina, it is very important to identify the regulatory mechanisms governing the contraction and relaxation of retinal pericytes.

L-Glutamate (L-Glu) is known as a relaxant of pericytes and Peppiatt et al. have reported that L-Glu treatment induces dilation of the retinal capillaries. Regarding the mechanism for the L-Glu-induced dilation of retinal capillaries, it has been proposed that L-Glu induces the production of nitric oxide (NO), a relaxant of the cells, and inhibits Ca\(^{2+}\) influx in retinal pericytes, thereby relaxing the pericytes. An in vitro analysis has indicated that this L-Glu-induced NO production occurs in the presence of L-Glu at a concentration of more than 1 \(\mu\)M. In addition, the dilation of retinal capillaries accompanied with pericytes was induced by treatment with 500 \(\mu\)M L-Glu. The compounds between the retinal interstitial fluid (ISF) and vitreous humor are considered to be freely exchangeable, and the concentration of L-Glu in the vitreous humor has been reported to be 444 \(\mu\)M in rats. Because the L-Glu level in the retinal ISF seems to be low compared with the concentration which induces dilation of the retinal capillaries and relaxation in the pericytes, it is possible that some mechanisms for concentrative L-Glu release are present in retinal pericytes and/or the cells surrounded by the pericytes. In neuronal cells, the packing of L-Glu into synaptic vesicles up to concentrations as high as 100 \(\mu\)M and exocytotic release of L-Glu in the vesicles into the synaptic cleft have been reported. It has also been reported that the uptake of L-Glu from the cytoplasm of neurons into synaptic vesicles involves the vesicular L-Glu transporters (VGLUTs) which belong to the solute carrier (SLC) family, such as VGLUT1/Slc17a7, VGLUT2/Slc17a6, and VGLUT3/Slc17a8. Recently, Mathur and Deutch have reported that VGLUT2 and VGLUT3 are expressed in brain microvaculature pericytes, suggesting that neural pericytes have the cellular machinery for exocytotic release of L-Glu. Regarding the supply of L-Glu in the cytoplasm before the packing of L-Glu into the vesicles of the cells in the brain, two processes have been considered: (i) L-Glu uptake from the extracellular fluid (ECF) into the cytoplasm and (ii) uptake of L-glutamine (L-Gln), a precursor of L-Glu, from the ECF, and then conversion to L-Glu via glutaminase (GA). However, there is no information at all about the expression of vesicular L-Glu transporters and L-Glu-synthesizing enzyme and the transport characteristics of L-Glu and L-Gln in retinal pericytes. It would be very useful to have more information about the L-Glu dynamics in retinal pericytes to better understand the role of L-Glu in the regulation of retinal capillary diameter.

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understand the manner of exocytotic l-Glu release, namely the use of l-Glu as an autocrine signal in retinal pericytes.

It has been shown that SLC families are involved in transport from ECF into the cells of l-Glu and l-Gln. As Na⁺-dependent transporters for acidic amino acids, such as d/l-l-Glu and d/l-α-aspartate, excitatory amino acid transporter (EAAT) 1/Slc1a3, EAAT2/Slc1a2, EAAT3/Slc1a1, EAAT4/Slc1a6, and EAAT5/Slc1a7 have been identified. It is known that the Na⁺-dependent transporters selectively recognizing the l-isomer of Glu and Asp are alanine–serine–cysteine transporter (ASCT) 1/Slc7a14 and ASCT2/Slc7a15,16,19,20 In addition, the Na⁺-independent and d/l-α-aspartate-insensitive system X⁺, which is composed of xCT/Slc7a11 and 4F2hc/Slc3a2, has been reported to be involved in the influx transport of l-Glu at the plasma membrane of cells.20–22 Regarding the plasma membrane transporters for l-Gln, ASCT2 accepts l-Gln, as a substrate, 23) although ASCT1 does not recognize l-Gln.24 Sodium-coupled neutral amino acid transporter (AT) 1/Slc38a1 and AT2/Slc38a2, which are referred to as system A, mediates Na⁺-dependent uptake of neutral amino acids such as l-Gln, l-alanine, and l-proline.25–27 SN1/Slc38a3 and SN2/Slc38a5, isoforms of system N, are also Na⁺-dependent transporters of l-Gln, l-asparagine, and l-histidine.28–30 System B₀,₅,⁺/uni2032 (ATB₀,₅/Slc6a14) and system y⁺/uni2032L (Y+/L amino acid transporter (y⁺LAT) 1/Slc7a7 and y⁺LAT2/Slc7a6) have reported to act as a neutral amino acid transporter in an Na⁺-dependent manner and also to recognize cationic amino acids.31,32 As an Na⁺-independent transporter for neutral amino acids including l-Gln, system y⁺/uni2032L (b₀,₅/uni2032AT/Slc7a9) and system L (LAT1/Slc7a5 and LAT2/Slc7a8) has been reported.33–35 In order to understand the dynamics of L-Glu and L-Gln in retinal pericytes, it is important to clarify the contribution of these plasma membrane transporters to the transport of l-Glu and l-Gln from the ECF to the cells.

The purpose of this study was to investigate the presence of l-Glu-storing systems in retinal pericytes. The expression of vesicular l-Glu transporters and l-Glu-synthesizing enzyme was elucidated by a reverse transcription-polymerase chain reaction (RT-PCR) in conditionally immortalized rat retinal pericytes (TR-rPCT1 cells).36,37 Moreover, the transport characteristics of l-Glu and l-Gln was evaluated by uptake studies using TR-rPCT1 cells.

MATERIALS AND METHODS

Animals Adult Wistar rats (ca.150g, male) were purchased from Japan SLC (Hamamatsu, Japan). They were maintained in a controlled environment and all experiments were approved by the Animal Care Committee, University of Toyama.

Reagents l-Glutamic acid, [³⁰⁸H]-l-Glu, 27.0 Ci/mmol and l-glutamine, l-[³⁰⁸H]-l-Gln, 49.8 Ci/mmol were purchased from Moravek Biochemicals (Brea, CA, U.S.A.). All other chemicals were of analytical grade and available commercially.

Cell Culture TR-rPCT1 cells, conditionally immortalized rat retinal pericytes, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 100 U/mL benzylpenicillin potassium, 100 µg/mL streptomycin and 10% fetal bovine serum (Moregate, Bulimba, Australia). The cells were maintained at 33°C, which is the permissive temperature at which the temperature-sensitive simian virus 40 large T-antigen is activated, in an atmosphere of 5% CO₂ in air.36

RT-PCR RT-PCR analysis was performed as described previously with minor modifications.38 Total RNA was prepared from TR-rPCT1 cells and rat tissues using an RNaseasy Mini kit (Qiagen, Hilden, Germany) and TRIZol reagent (Life Technologies, Carlsbad, CA, U.S.A.), respectively, according to the manufacturer’s protocol. Single-stranded cDNA was prepared from 1 µg total RNA using ReverTra Ace (Toyobo, Osaka, Japan) and oligo dT primer. PCR was performed using ExTaq DNA polymerase (TaKaRa Shuzo, Kyoto, Japan) with the following thermal cycle program: 1 cycle of 94°C for 30 s, and 30–40 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min. The RT-PCR of each sample RNA in the absence of reverse transcriptase was used as a negative control. The sequences of the primer sets are shown in Table 1. The RT-PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide (0.6 µg/mL) and visualized using ultraviolet light.

Table 1. Primer Sets Used for RT-PCR Analysis

<table>
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<th>Target</th>
<th>GenBank Accession No.</th>
<th>Nucleotide sequences (Upper, sense primer; Lower, antisense primer)</th>
<th>Product size (bp)</th>
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<td>VGLUT1</td>
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<td>5′-aagtgtgaggtgagc-3′</td>
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<td>VGLUT2</td>
<td>NM_053427</td>
<td>5′-gttacetcaaggtgacag-3′</td>
<td>209</td>
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<td>VGLUT3</td>
<td>NM_153725</td>
<td>5′-atcggagctctcctcctc-3′</td>
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<tr>
<td>LGA</td>
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<td>209</td>
</tr>
<tr>
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<td>461</td>
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<td>579</td>
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<tr>
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<td>5′-acctgtctctctctcatcagct-3′</td>
<td>335</td>
</tr>
<tr>
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<tr>
<td>EAAT4</td>
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<td>313</td>
</tr>
<tr>
<td>EAAT5</td>
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<td>587</td>
</tr>
<tr>
<td>ASCT2</td>
<td>NM_175758</td>
<td>5′-etcttcagagctcagctcagct-3′</td>
<td>388</td>
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</table>
period, uptake was terminated by removing the solution, and cells were rinsed three times with ice-cold ECF buffer. Then, the cells were solubilized in 1 M NaOH and subsequently neutralized with 1 M HCl. An aliquot was taken for measurement of the radioactivity and protein content using a liquid scintillation counter (LSC-5200; ALOKA, Tokyo, Japan) and a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, U.S.A.), respectively, with bovine serum albumin as a standard. The uptake of [3H]L-Glu or [3H]L-Gln was expressed as the cell/medium ratio (µL/mg protein, Eq. 1).

\[
\text{Cell/medium ratio (µL/mg protein)} = \frac{\text{[3H]-radioactivities in the cells (dpm/mg protein)}}{\text{[3H]-radioactivities in medium (dpm/µL)}}
\]  

In the analysis of the concentration-dependent uptake, the uptake data of L-Glu and L-Gln were fitted, using the non-linear least-square regression analysis program (MULTI),\(^3\) to a one saturable and one non-saturable process model as follows:

\[
V = V_{\text{max}} \times \frac{[S]}{(K_m + [S]) + K_d \times [S]} 
\]  

where \(V\) is the uptake rate of L-Glu or L-Gln, \(V_{\text{max}}\) is the maximum uptake rate of L-Glu or L-Gln, \([S]\) is the concentration of L-Glu or L-Gln, \(K_m\) is the corresponding Michaelis–Menten constant, and \(K_d\) is the non-saturable uptake rate constant.

RESULTS

mRNA Expression of Vesicular L-Glutamate Transporters and L-Glutamate-Synthesizing Enzymes in TR-rPCT1 Cells mRNA expression of VGLUT1–3 and two kinds of L-glutamate synthesizing enzymes, kidney-type glutaminase (KGA) and liver-type glutaminase (LGA),\(^4\) in TR-rPCT1 cells was examined by RT-PCR analysis (Fig. 1). RT-PCR gave amplified products at the expected sizes of 231 bp for VGLUT1 mRNAs, in TR-rPCT1 cells as well as in rat brain, a positive control (Fig. 1A). On the other hand, mRNA of VGLUT2 and VGLUT3 was detected in rat brain as a positive control, but not in TR-rPCT1 cells (Fig. 1A). Regarding the expression of L-glutamate-synthesizing enzymes (Fig. 1B), the amplified products at the expected sizes of 461 bp and 209 bp for KGA and LGA, respectively, were obtained. These results confirm the mRNA expression of VGLUT1, KGA, and LGA in TR-rPCT1 cells.

1-Glutamate Uptake by TR-rPCT1 Cells \([\text{[3H]}\) L-Glu uptake by TR-rPCT1 cells exhibited a time-dependent increase for at least 10 min with an initial uptake rate of 4.66±0.11 µL/(min·mg protein) (Fig. 2A) and a 66% decrease under Na\(^+\)-free conditions (Fig. 2B). Moreover, 1-Glu uptake was concentration-dependent and composed of saturable and non-saturable processes (Fig. 3). The apparent \(K_m\) and \(V_{\text{max}}\) of the saturable process were found to be 22.4±7.7 µM and 170±28 pmol/(min·mg protein), respectively, and the apparent \(K_d\) of the non-saturable process was 0.858±0.070 µL/(min·mg protein).

Fig. 1. RT-PCR Analysis of Subtypes of Vesicular L-Glutamate Transporter (VGLUT) (A) and Glutaminase (GA) (B) in TR-rPCT1 Cells
Rat brain and liver were used as the respective positive controls. + and − denote the presence and absence of reverse transcriptase, respectively.

Fig. 2. Time-Course (A) and Effect of Na\(^+\)-Free Condition (B) of \([\text{[3H]}\) L-Glu Uptake by TR-rPCT1 Cells
(A) [3H]-Glu uptake (0.5 µCi/mL, 19 nM) by TR-rPCT1 cells was performed at 37°C. Each point represents the mean±S.E.M. (n=3). (B) The [3H]-Glu uptake by TR-rPCT1 cells was performed at 37°C for 10 min in the presence (control) or absence (Na\(^+\)-free) of extracellular Na\(^+\). Each column represents the mean±S.E.M. (n=3). *p<0.01, significantly different from control.
The inhibition study using inhibitors of L-Glu transporters was performed to characterize the [3H]L-Glu transport mechanism(s) in TR-rPCT1 cells (Table 2). At a concentration of 1 mM, [3H]L-Glu uptake by TR-rPCT1 cells was significantly decreased by 85% and 48% in the presence of unlabeled L-Glu and L-aspartate, a substrate of EAATs and ASCT2, respectively. D-Aspartate, a substrate of EAATs, and L-cysteine, a substrate of EAAT3 and ASCT2, inhibited the [3H]L-Glu uptake by ca. 49%. Of ASCT2 substrates, L-alanine exhibited a significant inhibition of the [3H]L-Glu uptake by 40%, whereas L-serine had little effect. Kainic acid is a known inhibitor of EAAT2 and it inhibited [3H]L-Glu uptake by 27%. L-Histidine, a neutral amino acid, had no inhibitory effect on [3H]L-Glu uptake.

Table 2. Effect of Several Inhibitors on [3H]L-Glu Uptake by TR-rPCT1 Cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±3</td>
</tr>
<tr>
<td>L-Glu</td>
<td>15.0±1.2*</td>
</tr>
<tr>
<td>d-Aspartate</td>
<td>50.9±2.1*</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>51.8±2.2*</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>51.5±1.6*</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>60.2±1.3*</td>
</tr>
<tr>
<td>Kainate</td>
<td>73.2±5.7*</td>
</tr>
<tr>
<td>L-Serine</td>
<td>90.0±9.7</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>135±2*</td>
</tr>
</tbody>
</table>

Uptake of [3H]L-Glu (0.5 µCi/mL, 19 nM) by TR-rPCT1 cells was performed at 37°C for 10 min in the absence (control) or presence of inhibitors at the concentration of 1 mM. Each value represents the mean±S.E.M. (n=3–6). *p<0.01, significantly different from control.

The inhibition study using inhibitors of L-Glu transporters was performed to characterize the [3H]L-Glu transport mechanism(s) in TR-rPCT1 cells (Table 2). At a concentration of 1 mM, [3H]L-Glu uptake by TR-rPCT1 cells was significantly decreased by 85% and 48% in the presence of unlabeled L-Glu and L-aspartate, a substrate of EAATs and ASCT2, respectively. D-Aspartate, a substrate of EAATs, and L-cysteine, a substrate of EAAT3 and ASCT2, inhibited the [3H]L-Glu uptake by ca. 49%. Of ASCT2 substrates, L-alanine exhibited a significant inhibition of the [3H]L-Glu uptake by 40%, whereas L-serine had little effect. Kainic acid is a known inhibitor of EAAT2 and it inhibited [3H]L-Glu uptake by 27%. L-Histidine, a neutral amino acid, had no inhibitory effect on [3H]L-Glu uptake.

L-Glu Uptake by TR-rPCT1 Cells [3H]L-Glu uptake by TR-rPCT1 cells exhibited a time-dependent increase for at least 10 min with an initial uptake rate of 9.94±0.71 µL/(min·mg protein) (Fig. 4A). Under Na⁺-free conditions, the [3H]L-Glu uptake was significantly decreased by 81% (Fig. 4B). Moreover, the uptake of L-Glu exhibited a concentration dependence and was composed of saturable and non-saturable components. The apparent Kₘ and Vₘₐₓ of the saturable process were found to be 163±59 µM and 1.88±0.57 nmol/(min·mg protein), respectively, and the apparent Kₐ of the non-saturable process was 1.97±0.43 µL/(min·mg protein) (Fig. 5). Moreover, [3H]L-Glu uptake by TR-rPCT1 cells was inhibited by 79% in the presence of 1 mM unlabeled L-Glu and L-alanine, L-serine, L-cysteine, and L-threonine, which are substrates of ASCT2, inhibited it by more than 50% at a concentration of 1 mM (Table 3). L-Histidine (a substrate of SN1 and SN2), 2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid (BCH, an inhibitor of LAT1 and LAT2), and L-proline (a substrate of ATA1 and ATA2) at 1 mM produced approximately 25% inhibition of [3H]L-Glu uptake (Table 3). In contrast, L-arginine, which is a substrate of ATB¹⁻, b⁰⁺⁺AT, y¹LAT1, and y¹LAT2, had little effect on the [3H]L-Glu uptake (Table 3).

Expression of Plasma Membrane Transporters for L-Glu and L-Gln in TR-rPCT1 Cells To determine the expression
of transporters which are involved in l-Glu transport at the plasma membrane of retinal pericytes, RT-PCR analysis was performed with total RNA extracted from TR-rPCT1 cells and rat brain, as a positive control (Fig. 6). Bands indicative of EAAT1 and ASCT2 expression were detected in TR-rPCT1 cells and rat brain, but not in TR-rPCT1 cells.

**DISCUSSION**

In the present study, the expression of VGLUT1–3 in retinal pericytes was evaluated using TR-rPCT1 cells, which are an appropriate model having the characteristics of retinal pericytes as L-Glu. Therefore, the expression of these molecules implies the L-Glu autocrine action, and further functional studies of L-Glu synthesis in the cytoplasm and packing of L-Glu into the vesicles could help us to understand the physiological importance of these molecules.

To examine the characteristics of L-Glu transport from the ECF into retinal pericytes, which is the one of the ways of supplying L-Glu to the cytoplasm of retinal pericytes from the retinal ISF, L-Glu uptake was examined using TR-rPCT1 cells. The uptake of [3H]l-Glu was time-dependently increased up to at least 10 min (Fig. 2A). This L-Glu uptake occurred in both a saturable and non-saturable manner (Fig. 3). The uptake clearance of the saturable process ($V_{\text{max}}/K_m$) of L-Glu uptake by TR-rPCT1 cells was found to be 7.59 µL/(min·mg protein), which is 8.8-fold greater than that of the non-saturable process ($K_m = 0.858$ µL/(min·mg protein)) of the L-Glu uptake. This result indicates that carrier-mediated uptake of L-Glu plays a major role in L-Glu transport from the ECF into the retinal pericytes.

This L-Glu uptake by TR-rPCT1 cells for 10 min was significantly decreased by 66% in the absence of extracellular Na$^+$ (Fig. 2B). This result suggests the major involvement of Na$^+$-dependent L-Glu transporters, such as EAAT1–5 and ASCT1–2, in the L-Glu uptake by TR-rPCT1 cells although the Na$^+$-independent process of L-Glu uptake, which is considered to be mediated by system X$c^-$, cannot be completely negligible. The $K_m$ value for the saturable process of L-Glu uptake by TR-rPCT1 cells (22.4 µM, Fig. 3) is similar to that obtained for L-Glu influx transport via rat EAAT1–5 (EAAT1, 11 µM;
EAAT2, 17 µM; EAAT3, 12 µM; EAAT4, 1.3 µM; EAAT5, 61 µM. However, the K_m values of mouse system X^- and ASCT2 for L-Glu transport are 71- and 667-fold greater, respectively (mouse system X^-, K_m = 160 µM; mouse ASCT2, K_m = 1.6 mM), than that for the saturable process of L-Glu uptake by TR-rPCT1 cells. Taking these lines of evidence into consideration, it is implied that the saturable process of L-Glu uptake by TR-rPCT1 cells is carried out by EAAT1–5, and the non-saturable process could be conducted by system X^- and/or ASCT2. Moreover, [3H]L-Glu uptake by TR-rPCT1 cells was strongly inhibited by unlabeled L-Glu. It has been reported that l-aspartate and l-cysteine are substrates or inhibitors of EAATs and ASCT2 and inhibit system X^- mediated L-Glu transport at the concentration of 5 mM. At the concentration of 1 mM, [3H]L-Glu uptake was reduced by approximately 50% in the presence of l-aspartate and l-cysteine. n-Aspartate, which is a substrate of EAATs but not ASCTs and system X^-22), also inhibited [3H]L-Glu uptake by TR-rPCT1 cells by approximately 50%. These results imply that EAATs, but not ASCTs and system X^-22), mainly contribute to l-Glu uptake by retinal pericytes. Kainate is known as a substrate of EAAT249) and moderately inhibited [3H]l-Glu uptake by TR-rPCT1 cells compared with other EAAT inhibitors. Regarding the inhibitory effect of ASCT substrates,19), l-alanine inhibited [3H]l-Glu uptake by 40%, whereas l-serine had little effect. In addition, the uptake of [3H]l-Glu was not inhibited in the presence of l-histidine, which is not a substrate of EAAT, ASCT, and system X^-22). Taking all these observations into consideration, it is suggested that l-Glu uptake by TR-rPCT1 cells involves EAATs, but not ASCTs and system X^-22). Of the EAATs, mRNA expression of EAAT1 in TR-rPCT1 cells was confirmed by RT-PCR analysis, although mRNAs of EAAT2, EAAT3, EAAT4, and EAAT5 were not detected (Fig. 6). Consequently, these results suggest that EAAT1 is mainly involved in carrier-mediated l-Glu uptake by retinal pericytes.

In the present study, the influx transport of l-Gln, a precursor of l-Glu, at the plasma membrane of retinal pericytes was examined using TR-rPCT1 cells (Figs. 4–6) since TR-rPCT cells express the mRNA of KGA and LGA (Fig. 1). [3H]l-Gln uptake by TR-rPCT1 cells exhibited a time-dependent increase up to at least 10 min (Fig. 4A). This l-Gln uptake occurred in both a saturable and non-saturable manner (Fig. 5). The uptake clearance of the saturable process (V_max/K_m) of l-Gln uptake was found to be 11.5 µL/min·mg protein, which is 5.9-fold greater than that of the non-saturable process (K_m = 1.97 µL/min·mg protein) of l-Glu uptake. This result supports the major involvement of carrier-mediated processes in l-Gln influx transport in retinal pericytes.

It has been reported that l-Gln influx transport at the plasma membrane involves extracellular Na^+ -dependent transporters (i.e., ASCT2, y^+LAT1, 2, ATA1, 2, SNP1, 2, and ATB5, but not ASCT1) and Na^+ -independent transporters (i.e., b^0^v^AT, LAT1, 2). l-Gln uptake by TR-rPCT1 cells was significantly decreased in the absence of extracellular Na^+ (Fig. 4B), suggesting the major role of Na^+ -dependent transport processes in l-Gln uptake by retinal pericytes. The K_m value for a saturable process of l-Gln uptake by TR-rPCT1 cells (163 µM, Fig. 5) is comparable to the corresponding value of l-Gln transport via rat ASCT2 (70 µM), rat LAT2 (151 µM), rat y^+LAT2 (163 µM), and human ATA1 (498 µM), but not human ATA2 (1.65 mM) and human LAT1 (2.2 mM).23,26,27,32,34,35) In the inhibition study (Table 3), [3H]l-Gln uptake by TR-rPCT1 cells was significantly inhibited by more than 54% in the presence of ASCT2 substrates/inhibitors, such as l-alanine, l-cysteine, l-serine, and l-threonine, as well as unlabeled l-Gln.19,24) On the other hand, the inhibitory effect of substrates of SNP1, 2, LAT1, 2, and ATA1, 2, i.e., l-histidine, BCH, and l-proline, respectively,50,51) was not strong compared with that of ASCT2 substrates. l-Arginine, which has been reported as a substrate or inhibitor of ATB5, y^+LAT1, y^+LAT2, and b^0^v^AT,31–33) did not affect [3H]l-Glu uptake by TR-rPCT1 cells. Moreover, RT-PCR analysis showed that ASCT2 mRNA was detected in TR-rPCT1 cells (Fig. 6). Taking these results into consideration, it is suggested that ASCT2 plays a major role in l-Gln transport at the plasma membrane of retinal pericytes.

It has been reported that EAAT1 knockout mice exhibit an increased retinal level of l-Glu.52) In general, it is considered that Müller cells and retinal astrocytes, which are retinal glia, play a role in the elimination of l-Glu from the retinal ISF since EAAT1 is reported to be expressed in these glial cells.53) Our study suggests the involvement of EAAT1 in l-Glu transport at the plasma membrane of retinal pericytes (Figs. 2–3, 6, Table 2), implying the participation of retinal pericytes in l-Glu elimination from the retinal ISF by EAAT1-mediated l-Glu uptake in a similar manner to Müller cells and astrocytes. l-Gln in the retinal ISF is known to be released from Müller cells, and packed into synaptic vesicles as l-Glu after the conversion via glutaminases in the neuronal cells for concentrative release of l-Glu into the synaptic cleft.54) Since it was found that l-Gln was taken up into retinal pericytes via ASCT2 (Figs. 4–6, Table 3), it is considered that retinal pericytes modulate the l-Gln level in the retinal ISF by l-Gln transport into the cytoplasm and, thus, the synthesis and concentrative packaging of l-Glu in the retinal neurons. Taking these points into consideration, it is possible that EAAT1 and ASCT2 in retinal pericytes take part in the modulation of the level of l-Glu and l-Gln in the retinal ISF, and would significantly regulate the l-Glu level in the retinal ISF and neuroexcitatory responses in the retina.

In conclusion, EAAT1 and ASCT2 are involved in the influx transport of l-Glu and l-Gln at the plasma membrane of retinal pericytes. Moreover, mRNA expression of l-Glu-synthesizing enzymes, KGA and LGA, in retinal pericytes is indicated. These in vitro analyses implies that l-Gln, which is taken up into the cytoplasm of retinal pericytes, is converted to l-Glu via GA. Regarding the l-Glu autocrine action in retinal pericytes, it is proposed that l-Glu, which is derived from the retinal ISF as l-Glu and is synthesized by l-Gln, could be packed and concentrated in VGLUT1-expressing vesicles of retinal pericytes, and released into the retinal ISF in an exocytotic manner as required. Our findings provide insight into the physiological roles of retinal pericytes in the l-Glu-inducing responses in the retina.

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Conflict of Interest The authors declare no conflict of interest.
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