Amino Acid Residues Involved in the Substrate Specificity of TauT/SLC6A6 for Taurine and γ-Aminobutyric Acid

Tohru Yahara,a Masanori Tachikawa,a,b Shin-ichi Akanuma,a Yoshiyuki Kubo,a and Ken-ichi Hosoya,a,b

aDepartment of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; Toyama 930–0194, Japan; and bDivision of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University; Sendai 980–8578, Japan.

Received December 25, 2013; accepted March 4, 2014

Taurine transporter (TauT/SLC6A6) is an “honorary” γ-aminobutyric acid (GABA) transporter because of its low affinity for GABA. The sequence analysis of TauT implied the role of Gly57, Phe58, Leu306 and Glu406 in the substrate recognition of TauT, and amino acid substitutions were performed. Immunocytochemistry supported no marked effect of mutations on the expression of TauT. TauT-expressing oocytes showed a reduction in [3H]taurine uptake by G57E, F58I, L306Q and E406C, and change in [3H]GABA uptake by G57E and E406C, suggesting their significant roles in the function of TauT. G57E lost the activity of [3H]taurine and [3H]GABA uptake, suggesting that Gly57 is involved in the determination of substrate pocket volume and in the interaction with substrates. E406C exhibited a decrease and an increase in the affinity for taurine and GABA, respectively, suggesting the involvement of Glu406 in the substrate specificity of TauT. The inhibition study supported the role of Glu406 in the substrate specificity since [3H]taurine and [3H]GABA uptake by E406C was less sensitive to taurine and β-alanine, and more sensitive to GABA and nipeptic acid than was the case with wild type of TauT. F58I had an increased affinity for GABA, suggesting the involvement of Phe58 in the substrate accessibility. The kinetic parameters showed the decreased and increased affinities of L306Q for taurine and GABA, respectively, supporting that substrate recognition of TauT is conformationally regulated by the branched-side chain of Leu306. In conclusion, the present results suggest that these residues play important roles in the transport function and substrate specificity of TauT.

Key words taurine transporter; TauT (SLC6A6); γ-aminobutyric acid (GABA); GABA transporter (GAT); blood–retinal barrier (BRB)

In the retina, taurine (2-aminoethanesulfonic acid) play a role in the protection of the retinal neural cells by acting as an osmolyte and antioxidant.1–5) Recent progress in the research of membrane transporter and the blood–retinal barrier (BRB) has revealed the involvement of taurine transporter (TauT/SLC6A6) in taurine uptake by the retinal capillary endothelial cells and retinal glial cells (Müller cells) contributing to the regulation of retinal osmolarity.1,2,6–9)

Taurine is expressed in a variety of tissues, such as the retina, brain, liver, placenta and kidney.10,11) TauT transports taurine (Km = 43 μM) in a Na+- and Cl-–dependent manner. TauT also exhibits the transport activity for β-alanine (Km = 56 μM) and γ-aminobutyric acid (GABA) (Km = 1.5 mM), and a contribution by TauT has also been suggested to the transport of β-alanine and GABA across the inner BRB.5,12–14) Studies involving gene knockout mice have shown that TauT controls the concentration of taurine in various tissues since TauT gene knockout mice exhibited severe defects, such as retinal degeneration.15) The physiological importance of TauT is also supported by evidence that the expression of TauT is regulated by gene knockout mice have shown that TauT controls the concentration of taurine in various tissues since TauT gene knockout mice exhibited severe defects, such as retinal degeneration.15) The physiological importance of TauT is also supported by evidence that the expression of TauT is regulated by the extracellular taurine concentration and osmolarity.2,16–19) Only one of the known members of SLC6A, such as noradrenaline transporter (NET/SLC6A2), dopamine transporter (DAT/SLC6A3), serotonin transporter (SERT/SLC6A4) and creatine transporter (CRT/SLC6A8), play important roles in the central nervous system. GABA transporters (GATs), including GAT1/SLC6A1, GAT2/SLC6A13, GAT3/SLC6A11 and BGT1/SLC6A12, are involved in the transport of GABA by coupling with Na+ and Cl−, and exhibit a very similar amino acid sequence (>50%) to TauT11,21)

However, TauT exhibits a substrate specificity differing from GATs, and has been described as “honorary” GAT since TauT has a much lower affinity for GABA than GATs (<80 μM).14,22) Regarding the mechanisms, such as key amino acid residues, involved in their differences in substrate recognition, little is known apart from their well-characterized roles in physiology. Thus, it was our opinion that a study of the crystal structure of TauT and GATs would be helpful for investigating the mechanisms behind their differences in substrate specificity. However, detailed research into their crystal structure was expected to be difficult because of the difficulty in preparing sufficient quantities of mammalian membrane transporter proteins for three-dimensional crystal structure analysis.23)

In 2005, three-dimensional crystal structure of a bacterial homologue of SLC6A, LeuTAa, was reported to be very useful for the study of SLC6A.24) LeuTAa is a bacterial Na+-neurotransmitter symporter that transports L-leucine, and has twelve transmembrane (TM) domains. The crystal structure revealed that the substrate pocket of LeuTAa is formed by TM1, 3, 6 and 8, that exhibit a high degree of similarity to putatively corresponding TM domains of SLC6A members.24) In LeuTAa, these TM domains include the twelve amino acid residues, Asn21, Ala22, Leu25, Gly26, Val104, Tyr108, Phe252, Phe253, Ser256, Phe259, Ser355 and Ile359, that contribute to the substrate recognition of LeuTAa24) and the detailed study

* To whom correspondence should be addressed. e-mail: hosoyak@pha.u-toyama.ac.jp © 2014 The Pharmaceutical Society of Japan

© 2014 The Pharmaceutical Society of Japan
of LeuT<sub>Aa</sub> has contributed to updating the structure and functional residues of SLC6A. 25–29

Therefore, it would be helpful to use the information obtained in the study of LeuT<sub>Aa</sub> to investigate the mechanism for substrate recognition of TauT. In the present study, to investigate this mechanism, the amino acid residues of TauT were substituted with the corresponding residues of GATs, by referring to the functional residues of LeuT<sub>Aa</sub>, and the uptakes of taurine and GABA by TauT-expressing Xenopus laevis oocytes were determined.

MATERIALS AND METHODS

Reagents [2-3H]Taurine ([3H]taurine, 20 Ci/mmol) and 4-amino-<i>n</i>-[2,3-3H]butyric acid ([3H]GABA, 90 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.) and PerkinElmer Life and Analytical Sciences (Boston, MA, U.S.A.), respectively. Rabbit anti-rat TauT antibody and Alexa 488-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody were obtained from Alpha Diagnostic International (San Antonio, Texas, U.S.A.) and Life Technologies (Carlsbad, CA, U.S.A.), respectively. All other reagent-grade chemicals were commercially available.

Site-Directed Mutagenesis Site-directed mutagenesis was carried out as described previously. 30 The four mutants of TauT (wild type; WT), Gly57Glu (G57E), Phe58Ile (F58I), and Leu306Gln (L306Q) were generated by using the full-length cDNA of TauT (GenBank accession number NM_017206) cloned into the plasmid vector pGEM-HEN (pGEM-HEN/TauT) as a template. The pGEM-HEN plasmid constructs carrying the mutated TauT-cDNA were prepared by polymerase chain reaction (PCR) with a KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) and specific primers (Table I). The sequences of mutants were confirmed by means of an ABI PRIMS Genetic Analyzer (Life Technologies).

Uptake Study with TauT-Expressing Xenopus laevis Oocytes The uptake of [3H]taurine and [3H]GABA was studied using TauT-expressing oocytes, and cRNA-injected oocytes were prepared as described previously. 31 Briefly, pGEM-HEN vector harboring TauT cDNA were linearized by enzyme NotI and were used as templates in the capped cRNA synthesis by means of a RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI, U.S.A.). RNase-free water containing the capped cRNA (10 ng) was injected into oocytes. After 3 d of incubation in standard oocyte saline (SOS) solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM Na<sub>(2-hydroxyethyl)-</sub>piperazine-N<sub>2</sub>-2-ethanesulfonic acid (HEPES), 2.5 mM pyruvic acid, and 1% bovine serum albumin, pH 7.5) with antibiotics at 18°C, an uptake study was performed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.4) at 20°C, and was initiated by the application of 200 μL ND96 containing 0.5 μCi [3H]taurine (125 nm) or 0.5 μCi [3H]GABA (22.8 nm). After a designated time, uptake was terminated by adding ice-cold ND96 solution, and the oocytes were rinsed with ice-cold ND96 solution and then lysed with 5% sodium dodecyl sulfate (SDS). A liquid scintillation system LSC-7400 (Hitachi Aloka Medical, Tokyo, Japan) was used for the measurement of radioactivity.

Data Analysis Data analysis was carried out as described before. 32 In brief, the oocyte-to-medium (oocyte/medium) ratio was used to express the radio-labeled compound uptake by Xenopus laevis oocytes, using the Eq. 1:

\[
\text{oocyte/medium ratio} = \frac{([3H]\text{ dpm per oocyte})}{([3H]\text{ dpm per }\mu\text{L medium})}
\]

For the kinetic study of [3H]taurine and [3H]GABA transport, uptake data were fitted to a model described by Eq. 2 using the nonlinear least-square regression analysis program, MULTI. 33

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

where \(S\), \(V\), \(V_{\text{max}}\), and \(K_m\) are the concentration of compound, the uptake rate of the compound for 60 min, the maximal uptake rate and Michaelis–Menten constant, respectively.

Unless otherwise indicated, all data represent means±S.E.M. except for kinetic parameters. The data for the kinetic parameters represent means±S.D. For several groups, statistical significance of differences was determined by one-way analysis of variance followed by the modified Fisher’s least-significant difference method. For two groups, the significance of differences was determined by an unpaired, two-tailed Student’s <i>t</i>-test.

Immunohistochemical Analysis The frozen sections (20 μm in thickness) of TauT-expressing oocytes were prepared as described previously. 34,35 In brief, TauT-expressing oocytes were fixed with 4% paraformaldehyde in 10 mM phosphate buffer (8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.9 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2) for 3 h at room temperature, and immersed in 30% sucrose in ND96 solution. Oocytes were embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), and cut with a cryostat (Model CM1900; Leica Microsystems, Wetzlar, Germany) and then mounted onto silanized glass slides (Muto Pure Chemicals, Tokyo, Japan). The mounted sections were washed with phosphate-buffered saline containing 0.3% 

| Mutation | Nucleotide sequence | Position |
|----------|---------------------|----------|
| G57E     | Sense 5'-GAATTCGGTGGTCTGGCAATGTC-3' | 169–192  |
|          | Antisense 5'-TCAGCCGACAGCAGACAAAGT-3' | 146–168  |
| F58I     | Sense 5'-CATCGTGGGTTTGGCAATGTC-3' | 171–195  |
|          | Antisense 5'-CTGAGGATCTGGCTCCATGGA-3' | 147–170  |
| L306Q    | Sense 5'-AGGGGGGCATGACCTCAGGGAA-3' | 917–940  |
|          | Antisense 5'-GGGAGATJACGGTACGGAAAAATATC-3' | 913–939  |
| E406C    | Sense 5'-TTGTGCGAGGACAGCTACATCT-3' | 1215–1240 |
|          | Antisense 5'-AACAATTGGCTCTGGCCAGTGCAAGA-3' | 1190–1214 |
May 2014 819

Tween-20, and incubated with 10% goat serum (Nichirei, Tokyo, Japan) for 1 h at room temperature. Immunohistochemistry was performed as described previously, and rabbit anti-rat TauT antibody (1 µg/mL) and Alexa 488-labeled goat anti-rabbit IgG antibody (1:5000) were used as primary and secondary antibodies, respectively. Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.) was used to mount sections on coverslips, and confocal microscopy was performed using a TCS-SP5 instrument (Leica Microsystems).

RESULTS

Multiple Alignment Analysis of TauT, GATs and LeuT Aa
To identify the amino acid residues involved in the substrate specificity of TauT differing from GATs, their amino acid alignments were compared with that of TM1, 3, 6 and 8 in LeuT Aa (Fig. 1). As a result, Gly57, Phe58, Leu61, Gly62, Leu134, Tyr138, Phe299, Phe300, Ala303, Leu306, Ser402 and Glu406 of TauT were shown to correspond to the twelve important residues of LeuT Aa. Furthermore, comparison of the sequences of TauT and BGT1 revealed the four different residues at positions corresponding to Asn21, Ala22, Phe259 and Ile359 of LeuT Aa (Fig. 1), suggesting that Gly57, Phe58, Leu306 and Glu406 of TauT and Glu66, Ile67, Gln313 and Cys413 of BGT1 are possible determinants of their differences in substrate specificity. In addition, multiple alignment analysis revealed that Gly57, Phe58, Leu306 and Glu406 are conserved between human, mouse, rat, dog and monkey orthologs of TauT (data not shown).

Expression Analysis of Mutated TauT Proteins
According to the results obtained in the multiple alignments (Fig. 1), Gly57, Phe58, Leu306 and Glu406 of TauT were suggested as possible residues causing the differences in substrate recognition from GATs. Thus, to investigate the contributions of these residues to the function of TauT, Gly-to-Glu, Phe-to-Ile, Leu-to-Gln and Glu-to-Cys substitutions were performed at position 57, 58, 306 and 406 of TauT, respectively. In the confocal microscopy study with an anti-TauT antibody, the fluorescence

---

Fig. 1. Multiple Alignment of TauT, GATs and LeuT Aa. TauT (NP_058902.1), rGAT1 (NP_077347.1), rGAT2 (NP_598307.1), rGAT3 (NP_077348.1) and rBGT1 (NP_059031.1) were aligned with TM Domains 1, 3, 6 and 8 of A. aeolicus LeuT (LeuT Aa) (NP_214423.1)

The numbers refer to positions in the rat TauT sequence. The filled circles indicate the positions of residues in LeuT Aa reported to interact with the substrate, L-leucine. Residues conserved between TauT and BGT1 are boxed in gray. Differences between the TauT and rBGT1 are shown in white and boxed in black. The open squares show residues involved in coordinating sodium ions.

---

Fig. 2. Expression of TauT Proteins in Oocytes
Confocal microscopy was performed with an anti-TauT antibody raised against the C-terminus of TauT protein. Arrow head indicates TauT proteins expressed at the membrane of oocytes. Scale bars: 50 µm.
signal of TauT (WT, G57E, F58I, L306Q and E406C) proteins were detected at the membrane of oocytes, and there was no marked difference in their amounts of protein expression (Fig. 2). No signal was observed in water-injected oocytes.

**Uptake of \[^3\text{H}\]Taurine and \[^3\text{H}\]GABA by TauT-Expressing Oocytes** To investigate the function of TauT-mutants, the uptake study of \[^3\text{H}\]taurine and \[^3\text{H}\]GABA was performed with cRNA-injected oocytes. Oocytes expressing WT and mutants of TauT, except for G57E, exhibited time-dependent increases in \[^3\text{H}\]taurine and \[^3\text{H}\]GABA uptake for at least 60 min (Fig. 3).

G57E-expressing oocytes exhibited a marked reduction in \[^3\text{H}\]taurine and \[^3\text{H}\]GABA uptake by 99.8% and 91.8%, respectively, compared with WT-expressing oocytes, and the loss of TauT function was suggested to be caused by the Gly-to-Glu substitution at position 57 (Fig. 4). F58I-, L306Q- and E406C-expressing oocytes exhibited a significant reduction in \[^3\text{H}\]taurine uptake by 58.1%, 97.3% and 49.0%, respectively (Fig. 4A). The uptake of \[^3\text{H}\]GABA by E406C-expressing oocytes exhibited a significant increase (3.7-fold) compared with WT, while no significant change was exhibited by F58I and L306Q-expressing oocytes (Fig. 4B).

**Concentration-Dependent Taurine Uptake by TauT-Expressing Oocytes** To examine the concentration-dependence of the mutated TauT, data obtained in the uptake study were analyzed by Michaelis–Menten kinetics. WT-, F58I-, L306Q- and E406C-expressing oocytes exhibited a concentration-dependent uptake of taurine (Figs. 5A, C, E), with \(K_m\) values of 25.9±8.1, 27.4±3.2, 41.6±13.5 and 82.4±47.3 \(\mu\text{M}\), and \(V_{\text{max}}\) values of 198±25, 115±9, 5.27±1.23 and 183±56 pmol/(h·oocyte), showing a marked increase in the \(K_m\) value caused by the Glu-to-Cys substitution at position 406 (Table 2). The uptake clearances \((V_{\text{max}}/K_m)\) for taurine were estimated to be 7.68, 4.21, 0.127 and 2.22 \(\mu\text{L}/(h\cdot\text{oocyte})\), showing a markedly reduced uptake clearance caused by the Leu-to-Gln substitution at position 306 (Table 2).

**Concentration-Dependent GABA Uptake by TauT-Expressing Oocytes** WT-, F58I-, L306Q- and E406C-expressing oocytes exhibited a concentration-dependent uptake of GABA (Figs. 5B, D, F), with \(K_m\) values of 564±62, 103±9, 22.6±8.6 and 292±52 \(\mu\text{M}\), and \(V_{\text{max}}\) values of 338±22, 77.5±3.2, 8.27±1.23 and 628±61 pmol/(h·oocyte), showing a marked reduction in the \(K_m\) caused by the Leu-to-Gln substitution at position 306 (Table 2). Their uptake clearances

---

**Fig. 3. Time–Course Uptake of \[^3\text{H}\]Taurine and \[^3\text{H}\]GABA by TauT-Expressing Oocytes**
The uptake studies were performed for WT and TauT-mutants. The uptake of \[^3\text{H}\]taurine (125 nM) (A–C) and \[^3\text{H}\]GABA (28 nM) (D–F) by F58I- (A, D), L306Q- (B, E) and E406C- (C, F) expressing oocytes was examined at 20°C. Each symbol represents water (open diamond), wild type cRNA- (open circle) and mutant cRNA- (closed circle) injected oocytes. Each point represents the mean±S.E.M. (n=9–15).
uptake of [3H]taurine by TauT while it significantly inhibited the uptake of [3H]GABA, and it is reported that TauT has higher and lower affinities for taurine ($K_m$ = 43 µM) and GABA ($K_m$ = 1.46 mM) than that for GAA ($K_m$ = 215 µM), respectively.34,36 Supporting that the different inhibitory effect observed was caused the different affinities of TauT for substrates.

**DISCUSSION**

TauT (SLC6A6), a member of the SLC6A family, is a Na$^+$- and Cl$^-$-dependent taurine transporter, and its physiological relevance has been suggested in a number of in vivo and in vitro studies.1–4,15 However, little is known about the mechanism underlying the differences in substrate specificity between TauT and GATs in term of their sequence similarity.14,23 The study of LeuTα, a bacterial homologue of SLC6A6, showed the role of amino acid residues in its function,24 and the sequence analysis suggested that the four residues in TauT, Gly57, Phe58, Leu306 and Glu406, corresponded to Asn21, Ala22, Phe259 and Ile359 in LeuTα (Fig. 1), showing their possible roles in the substrate specificity of TauT. In this study, the amino acid substitution and functional analysis were performed to investigate the role of residues in the substrate recognition of TauT (Table 1).

The confocal microscopy suggested that the amino acid substitutions have no marked effect on the expression of TauT-mutant proteins since the specific signals for G57E, F58I, L306Q and E406C proteins were observed at the surface membrane of oocytes (Fig. 2). The [3H]taurine uptake study with TauT-expressing oocytes suggested contributions of Gly57, Phe58, Leu306 and Glu406 to taurine transport by TauT because of the significantly reduced uptake of [3H]taurine by G57E- and E406C-expressing oocytes (Figs. 3, 4). The study of [3H]GABA uptake suggested the contribution of Gly57 and Glu406 to GABA transport by TauT since G57E and E406C-expressing oocytes exhibited significant changes in [3H]GABA uptake (Figs. 3, 4).

G57E lost the activity of [3H]taurine and [3H]GABA uptake (Fig. 4), suggesting the role of Gly57 in the uptake function of TauT. It has been reported that TM1 of LeuTα binds to the substrate via the main chain oxygen of Asn21, and that its side chain acts as a volume determinant of the substrate pocket.24 The model simulation for hGAT1 showed a hydrogen bonding between GABA and the main chain oxygen of Tyr60 that corresponds to the Asn21 of LeuTα.26,27 Therefore, regarding G57E of TauT, the reduced uptakes were caused by the Gly-to-E mutation between GABA and the main chain oxygen of Tyr60 that corresponds to the Asn21 of LeuTα.26,27 Therefore, regarding G57E of TauT, the reduced uptakes were caused by the Gly-to-E mutation in TauT. It has been reported that TM1 of LeuTα binds to the substrate via the main chain oxygen of Asn21, and that its side chain acts as a volume determinant of the substrate pocket.24
has been reported to interact with the α-amino group of l-leucine.24) The involvement of the side chain of Phe58 was also supported by the substrate recognition of TauT since the side chain of Ala77 in hDAT1, corresponding to the Ala22 of LeuTαa, has been reported to be involved in the substrate recognition of hDAT1.28,29) The kinetic parameters suggest the involvement of Phe58 in the GABA recognition by TauT since F58I showed a higher affinity (\(K_m = 103 \mu M\)) for GABA than that of WT (\(K_m = 564 \mu M\)) (Table 2, Fig. 5). The structure formulae suggest a bulkier molecular size of GABA than that of taurine, supporting the hypothesis that the Phe-to-Ile substitution reduced the bulkiness of the side chain, thereby improving the accessibility of GABA to the substrate pocket. Thus, it is suggested that Phe58, putatively located in TM1 of TauT, is involved in the accessibility of the substrate to the substrate pocket. Furthermore, Ala22 of LeuTαa has been reported to

Table 2. Kinetic Parameters of Mutated-TauT for Taurine and GABA

| Mutants | Taurine | GABA |
|---------|---------|------|
|         | \(K_m\) (\(\mu M\)) | \(V_{max}\) (pmol/(h·oocyte)) | \(V_{max}/K_m\) (\(\mu L/(h·oocyte)\)) | \(K_m\) (\(\mu M\)) | \(V_{max}\) (pmol/(h·oocyte)) | \(V_{max}/K_m\) (\(\mu L/(h·oocyte)\)) |
| Wild-type | 25.9±8.1 | 198±25 | 7.68 | 564±62 | 338±22 | 0.599 |
| G57E | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| F58I | 27.4±3.2 | 115±9 | 4.21 | 103±9 | 77.5±3.2 | 0.749 |
| L306Q | 41.6±13.5 | 5.27±1.23 | 0.127 | 22.6±8.6 | 8.27±1.23 | 0.365 |
| E406C | 82.4±47.3 | 183±56 | 2.22 | 292±52 | 628±61 | 2.15 |

\(K_m\) and \(V_{max}\) values were estimated from the data obtained in the study of concentration-dependence (Fig. 5), and each value represents means±S.D. N.D., not detectable.
be a residue forming the Na\(^+\)-binding site that is essential for the function of LeuT\(_{\text{Aa}}\)^{24,37} and the insignificant change in \([^3\text{H}]\text{taurine}\) uptake of E406C strongly suggests a minor contribution of Phe58 in the Na\(^+\)-binding of TauT (Figs. 3D, 4B).

In TM6 of LeuT\(_{\text{Aa}}\), the side chain of Phe259 has been reported to form a hydrophobic interaction with the isopropyl-group of \(\text{i-leucine}^{24,29}\) and the multiple alignments suggest that Phe259 of LeuT\(_{\text{Aa}}\) corresponds to Leu306 of TauT (Fig. 1), implying a role of the side chain of Leu306 in the substrate recognition of TauT. In actual fact, the uptake study suggests that Leu306 contributes to the taurine transport of TauT since L306Q showed a significant reduction in \([^3\text{H}]\text{taurine}\) uptake by 97% while there was no significant effect on \([^3\text{H}]\text{GABA}\) uptake (Fig. 4). This is also supported by the transport clearance (\(V_{\text{max}}/K_m\)) of L306Q for taurine that is 60.5-fold lower than that of WT (Table 2). The kinetic parameters revealed the contribution of Leu306 to the substrate pocket of TauT since the Leu-to-Gln substitution showed lower and higher affinities, respectively, for taurine (\(K_m=41.6 \mu M\)) and GABA (\(K_m=22.6 \mu M\)) than those of WT (Table 2, Fig. 5). Leu has a bulkier side chain than that of Gln, and the Leu-to-Gln substitution caused the increased and decreased affinities for GABA and taurine, respectively, showing a minor contribution of the side chain bulkiness at position 306. The structural difference, the branched-side chain of Leu and the straight-side chain of Gln, suggests that the branched-side chain of Leu306 conformationally determines the volume of the substrate pocket of TauT.

Glu406 of TauT is suggested to correspond to Ile359 localized in TM8 of LeuT\(_{\text{Aa}}\) (Fig. 1), and the side chain of Ile359 in LeuT\(_{\text{Aa}}\) has been reported to form a hydrophobic interaction with \(\text{i-leucine}^{24}\) implying the involvement of Glu406 in the substrate recognition of TauT. The uptake study suggests the contribution of Glu406 to the \([^3\text{H}]\text{taurine}\) and \([^3\text{H}]\text{GABA}\) transport by TauT since E406C exhibited significantly lower and greater uptake of \([^3\text{H}]\text{taurine}\) and \([^3\text{H}]\text{GABA}\), respectively, than those of WT (Fig. 4). This is also supported by the 3.5-fold lower and 3.6-fold higher transport clearance (\(V_{\text{max}}/K_m\)) values of E406C for taurine and GABA, respectively, than those of WT (Table 2). The kinetic parameters suggested the involvement of Glu406 in the substrate recognition of TauT since E406C showed lower and higher affinities for taurine and GABA, respectively, than those of WT (Table 2). Regarding \([^3\text{H}]\text{taurine}\) transport, E406C showed its GATs-like transport property since the Glu-to-Cys substitution exhibited a lower

### Table 3. Effect of Several Compounds on \([^3\text{H}]\text{Taurine}\) Uptake by TauT-Expressing Oocytes

| Inhibitors   | Concentration (\(\mu M\)) | Relative uptake (% of control) |
|-------------|---------------------------|-------------------------------|
|             |                           | Wild-type                     | E406C                        |
| Control     |                           | 100±8                         | 100±15                       |
| Taurine     | 50                        | 38.3±3.7*                     | 62.7±6.3                     |
| \(\beta\)-Alanine | 50                  | 61.7±5.6*                     | 153±28                       |
| GABA        | 1000                      | 32.4±6.3*                     | 5.08±0.86*                   |
| Nippecotic acid | 2000                | 65.3±11.8**                   | 3.57±0.87*                   |
| Betaine     | 2000                      | 82.4±13.4                     | 73.1±17.3                    |
| GAA         | 2000                      | 70.8±11.7                     | 65.3±12.0                    |

\([^3\text{H}]\text{Taurine}\) uptake by TauT-expressing oocytes was performed in the absence (control) or presence of inhibitors at 20°C for 60 min. Each value represents means±S.E.M. (n=14-50). *p<0.01, **p<0.05, significantly different from the control. GABA, \(\gamma\)-aminobutyric acid; GAA, guanidinoacetic acid.

### Table 4. Effect of Several Compounds on \([^3\text{H}]\text{GABA}\) Uptake by TauT-Expressing Oocytes

| Inhibitors   | Concentration (\(\mu M\)) | Relative uptake (% of control) |
|-------------|---------------------------|-------------------------------|
|             |                           | Wild-type                     | E406C                        |
| Control     |                           | 100±11                        | 100±11                       |
| Taurine     | 10                        | 40.3±9.2*                     | 104±13                       |
| \(\beta\)-Alanine | 10                | 55.1±13.2**                   | 113±15                       |
| GABA        | 500                       | 43.5±5.7*                     | 27.4±3.9*                    |
| Nippecotic acid | 500                | 43.8±9.1*                     | 13.8±1.7*                    |
| Betaine     | 500                       | 129±14                        | 91.8±11.7                    |
| GAA         | 2000                      | 36.2±4.9*                     | 45.1±8.5*                    |

\([^3\text{H}]\text{GABA}\) uptake by TauT-expressing oocytes was performed in the absence (control) or presence of inhibitors at 20°C for 60 min. Each value represents means±S.E.M. (n=13-30). *p<0.01, **p<0.05, significantly different from the control. GABA, \(\gamma\)-aminobutyric acid; GAA, guanidinoacetic acid.
sensitivity to taurine and β-alanine, and a higher sensitivity to GABA and nipecotic acid than WT (Table 3). These results suggest the involvement of the amino acid residue at position 406 in determining the substrate specificity of TauT, and this is also supported by the alteration of sensitivities to taurine, β-alanine, GABA and nipecotic acid observed in the transport of [3H]GABA by E406C (Table 4). Although amino acid substitution was performed by referring the sequence of rGT1, the Glu-to-Cys substitution caused no change in the sensitivity of TauT to betaine (Tables 3, 4). Thus, the minor contribution of Cys413 of rGT1, corresponding to Glu406 of TauT, was suggested for the recognition of betaine, and this is also supported by the conserved Cys between GATs (Fig. 1).

In conclusion, the sequence analysis showed that Gly57, Phe58, Leu306 and Glu406 of TauT are candidate residues determining the substrate specificity of TauT. The uptake study with TauT-mutants suggested their contribution to the substrate specificity of TauT. In particular, it was suggested that Phe58 and Leu306 are involved in the substrate accessibility and recognition of TauT, respectively, and that Glu406 is involved in the substrate specificity of TauT differing from that of GATs. These findings provide helpful information to increase our understanding of the transport function and role of TauT in the body.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for Promotion of Science, and the Nakatomi Foundation of Japan.

REFERENCES

1) Tomi M, Terayama T, Isobe T, Egami F, Morito A, Kusuhara M, Ohbuchi S, Kang YS, Terasaki T, Hosoya K. Function and regulation of taurine transporter at the inner blood–retinal barrier. Microvasc. Res., 73, 100–106 (2007).
2) Ando D, Kubo Y, Akanuma S, Yoneyama D, Tachikawa M, Hosoya K. Function and regulation of taurine transporter in Müller cells under osmotic stress. Neurochem. Int., 50, 597–604 (2012).
3) Miyamoto Y, Kulasvile P, Leibach FH, Ganapathy V. Taurine uptake in apical membrane vesicles from the bovine retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci., 32, 2542–2551 (1991).
4) El-Sherbiny A, Naggar H, Miyauuchi S, Ola MS, Maddox DM, Martin PM, Ganapathy V, Smith SB. Osmoregulation of taurine transporter function and expression in retinal pigment epithelial, glial, and Müller cells. Invest. Ophthalmol. Vis. Sci., 45, 694–701 (2004).
5) Torquatt, P, Alm A. Carrier-mediated transport of amino acids through the blood-retinal and blood-brain barriers. Graefe’s Arch. Clin. Exp. Ophthalmol., 224, 21–25 (1986).
6) Uchiha S, Kwon HM, Yamauchi A, Preston AS, Marumo F, Handler JS. Molecular cloning of the cDNA for an MDCK cell Na'- and Cl'-dependent taurine transporter that is regulated by hypertonicity. Proc. Natl. Acad. Sci. U.S.A., 89, 8230–8234 (1992).
7) Smith KE, Borden LA, Wang CH, Hartig PR, Brancheck TA, Weinschank RL. Cloning and expression of a high affinity taurine transporter from rat brain. Mol. Pharmacol., 42, 563–569 (1992).
8) Hosoya K, Tomi M, Tachikawa M. Strategies for therapy of retinal diseases using systemic drug delivery: relevance of transporters at the blood–retinal barrier. Expert Opin. Drug Deliv., 8, 1571–1587 (2011).
9) Tomi M, Hosoya K. The role of blood–ocular barrier transporters in retinal drug disposition: an overview. Expert Opin. Drug Metab. Toxicol., 6, 1111–1124 (2010).
10) Ramamoorthy S, Leibach FH, Mahesh VB, Han H, Yang-Feng T, Blakey RD, Ganapathy V. Functional characterization and chromosomal localization of a cloned taurine transporter from human placenta. Biochem. J., 300, 893–900 (1994).
11) Chen NH, Reith ME, Quick MW. Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. Pflugers Arch., 447, 519–531 (2004).
12) Liu OR, Lopez-Corcuera B, Nelson H, Mandiyan S, Nelson N. Cloning and expression of a cDNA encoding the transporter of taurine and beta-alanine in mouse brain. Proc. Natl. Acad. Sci. U.S.A., 89, 12145–12149 (1992).
13) Usui T, Kubo Y, Akanuma S, Hosoya K, β-Alanine and L-histidine transport across the inner blood–retinal barrier: Potential involvement in l-carnosine supply. Exp. Eye Res., 113, 135–142 (2013).
14) Tomi M, Tajima A, Tachikawa M, Hosoya K. Function of taurine transporter (Slc6a6/TauT) as a GABA transporting protein and its relevance to GABA transport in rat retinal capillary endothelial cells. Biochim. Biophys. Acta, 1778, 2138–2142 (2008).
15) Heller-Stib B, van Roeyen C, Rascher K, Hartwig HG, Huth A, Seeleiger MW, Wurskult U, Häussinger D. Disruption of the taurine transporter gene (taut) leads to retinal degeneration in mice. FASEB J., 16, 231–233 (2002).
16) Han X, Budreau AM, Chesney RW. Adaptive regulation of MDCK cell taurine transporter (pNCT) mRNA: transcription of pNCT gene is regulated by external taurine concentration. Biochim. Biophys. Acta, 1351, 296–304 (1997).
17) Takeuchi K, Toyohara H, Sakaguchi M. A hyperosmotic stress-induced mRNA of carp cell encodes Na' - and Cl'–dependent high affinity taurine transporter. Biochim. Biophys. Acta, 1464, 219–230 (2000).
18) Ito T, Fujio Y, Hirata M, Takatan T, Matsuda T, Muraoka S, Takanashi K, Azuma J. Expression of taurine transporter is regulated through the TonE (tonicity-responsive element)/TonEBP (TonE-binding protein) pathway and contributes to cytoprotection in HepG2 cells. Biochem. J., 382, 177–182 (2004).
19) Jayanthi LD, Ramamoorthy S, Mahesh VB, Leibach FH, Ganapathy V. Substrate-specific regulation of the taurine transporter in human placental choriocarcinoma cells (JAR). Biochim. Biophys. Acta, 1235, 351–360 (1995).
20) Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. Nat. Rev. Neurosci., 4, 13–25 (2003).
21) Takanaga H, Ohhtsuki S, Hosoya K, Terasaki T, GAT2/BGT-1 as a system responsible for the transport of gamma-aminobutyric acid at the mouse blood–brain barrier. J. Cereb. Blood Flow Metab., 21, 1232–1239 (2001).
22) Borden LA, GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem. Int., 29, 335–356 (1996).
23) Tate CG. Overexpression of mammalian integral membrane proteins for structural studies. FEBS Lett., 504, 94–98 (2011).
24) Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na'/Cl'–dependent neurotransmitter transporters. Nature, 437, 215–223 (2005).
25) Gabrielsen M, Sylte I, Dahl SG, Ravna AW. A short update on the structure of drug binding sites on neurotransmitter transporters. BMC Res. Notes, 4, 559–563 (2011).
26) Palló A, Bencsura A, Heja L, Beke T, Percez A, Kardos J, Simon A. Major human gamma-aminobutyrate transporter: in silico prediction of substrate efficacy. Biochim. Biophys. Res. Commun., 364, 952–958 (2007).
27) Wein T, Wanner KT. Generation of a 3D model for human GABA transporter hGAT-1 using molecular modeling and investigation of the binding of GABA. J. Mol. Model., 16, 155–161 (2010).
28) Beuming T, Kniazzeff J, Bergmann ML, Shi L, Gracia L, Ranisze-
May 2014

wska K, Newman AH, Javitch JA, Weinstein H, Gether U, Loland CJ. The binding sites for cocaine and dopamine in the dopamine transporter overlap. Nat. Neurosci., 11, 780–789 (2008).

29) Xhaard H, Backström V, Denessiouk K, Johnson MS. Coordination of Na⁺ by monoamine ligands in dopamine, norepinephrine, and serotonin transporters. J. Chem. Inf. Model., 48, 1423–1437 (2008).

30) Matsuyama R, Tomi M, Akanuma S, Tabuchi A, Kubo Y, Tachikawa M, Hosoya K. Up-regulation of L-type amino acid transporter 1 (LAT1) in cultured rat retinal capillary endothelial cells in response to glucose deprivation. Drug Metab. Pharmacokinet., 27, 317–324 (2012).

31) Tachikawa M, Ikeda S, Fujinawa J, Hirose S, Akanuma S, Hosoya K. γ-Aminobutyric acid transporter 2 mediates the hepatic uptake of guanidinoacetate, the creatine biosynthetic precursor, in rats. PLoS ONE, 7, e32557 (2012).

32) Tachikawa M, Tsuji K, Yokoyama R, Higuchi T, Ozeki G, Yashiki A, Akanuma S, Hayashi K, Nishura A, Hosoya K. A clearance system for prostaglandin D₂, a sleep-promoting factor, in cerebrospinal fluid: role of the blood–cerebrospinal barrier transporters. J. Pharmacol. Exp. Ther., 343, 608–616 (2012).

33) Yamaoka K, Tanigawara Y, Nakagawa T, Uno T. A pharmacokinetic analysis program (multi) for microcomputer. J. Pharmacobiodyn., 4, 879–885 (1981).

34) El-Jouni W, Jang B, Haun S, Machaca K. Calcium signaling differentiation during Xenopus oocyte maturation. Dev. Biol., 288, 514–525 (2005).

35) Kuwahara M, Asai T, Sato K, Shinbo I, Terada Y, Marumo F, Sasaki S. Functional characterization of a water channel of the nematode Caenorhabditis elegans. Biochim. Biophys. Acta, 1517, 107–112 (2000).

36) Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya K. The blood–brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. J. Neurochem., 111, 499–509 (2009).

37) Shi L, Quick M, Zhao Y, Weinstein H, Javitch JA. The mechanism of a neurotransmitter: sodium symporter-inward release of Na⁺ and substrate is triggered by substrate in a second binding site. Mol. Cell, 30, 667–677 (2008).

38) Ben-Yona A, Kanner BI. Transmembrane domain 8 of the γ-aminobutyric acid transporter GAT-1 lines a cytoplasmic accessibility pathway into its binding pocket. J. Biol. Chem., 284, 9727–9732 (2009).

39) Ben-Yona A, Bendahan A, Kanner BI. A glutamine residue conserved in the neurotransmitter: Sodium Symporters is essential for the interaction of chloride with the GABA transporter GAT-1. J. Biol. Chem., 286, 2826–2833 (2011).