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

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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 [^{3}H]taurine uptake by G57E, F58I, L306Q and E406C, and change in [^{3}H]GABA uptake by G57E and E406C, suggesting their significant roles in the function of TauT. G57E lost the activity of [^{3}H]taurine and [^{3}H]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 [^{3}H]taurine and [^{3}H]GABA uptake by E406C was less sensitive to taurine and β-alanine, and more sensitive to GABA and nipecotic 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]

TauT is expressed in a variety of tissues, such as the retina, brain, liver, placenta and kidney.[10,11] TauT transports taurine (K_m=43 μM) in a Na^+- and Cl^--dependent manner. TauT also exhibits the transport activity for β-alanine (K_m=56 μM) and γ-aminobutyric acid (GABA) (K_m=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 the extracellular taurine concentration and osmolarity.[2,16–19] TauT belongs to the SLC6A family that includes Na^+- and Cl^--dependent neurotransmitter transporters,[11,20] and it is known that 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 TauT.[11,21]

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, $LeuT_{Aa}$, was reported to be very useful for the study of SLC6A.[24] $LeuT_{Aa}$ 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 $LeuT_{Aa}$ 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 $LeuT_{Aa}$, 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 $LeuT_{Aa}$,[24] and the detailed study

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of $LeuT_{Aa}$ 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 $LeuT_{Aa}$ to investigate the mechanism for substrate recognition of TauT. In the present study, to investigate this mechanism, the amino acid resides of TauT were substituted with the corresponding residues of GATs, by referring to the functional residues of $LeuT_{Aa}$, and the uptakes of taurine and GABA by TauT-expressing *Xenopus laevis* oocytes were determined.

MATERIALS AND METHODS

Reagents [2-^{3}H]Taurine ([^{3}H]taurine, 20 Ci/mmol) and 4-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]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), Leu306Gln (L306Q) and Glu406Cys (E406C), 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 1). 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 [^{3}H]taurine and [^{3}H]GABA was studied using TauT-expressing oocytes, and cRNA-injected oocytes were prepared as described elsewhere.[31)] Briefly, pGEM-HEN vector harboring TauT cDNA were linearized by enzyme digestion with *Not*I or *Nhe*I, 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 $CaCl_2$, 1 mM $MgCl_2$, 5 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-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 $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) at 20°C, and was initiated by the application of 200 μL ND96 containing 0.5 μCi [^{3}H]taurine (125 nM) or 0.5 μCi [^{3}H]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:

$$\text{oocyte/medium ratio} = ([^3\text{H}]\ \text{dpm per oocyte}) / ([^3\text{H}]\ \text{dpm per}\ \mu\text{L medium}) \quad (1)$$

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

$$V = (V_{max} \times S) / (K_m + S) \quad (2)$$

where S, V, V_{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 *t*-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_2HPO_4, 1.9 mM NaH_2PO_4, 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%

Table 1. Primers for the Site-Directed Mutagenesis of TauT

Mutation		Nucleotide sequence	Position
G57E	Sense	5′-GAATTCGTGGGTTTGGGCAATGTC-3′	169–192
	Antisense	5′-TCCGGCCACAGACAGCACAAAGT-3′	146–168
F58I	Sense	5′-CATCGTGGGTTTGGGCAATGTCTGG-3′	171–195
	Antisense	5′-CCTCCGGCCACAGACAGCACAAAG-3′	147–170
L306Q	Sense	5′-AGGGGGCCATGACCTCACTGGGAA-3′	917–940
	Antisense	5′-GGCAGATAGCGTAGGAAAAGAATATCT-3′	913–939
E406C	Sense	5′-TTGTGTCGAAGGACAGATCACATCCT-3′	1215–1240
	Antisense	5′-ACAAACTGGCTGTCCAGTCCAAGCA-3′	1190–1214

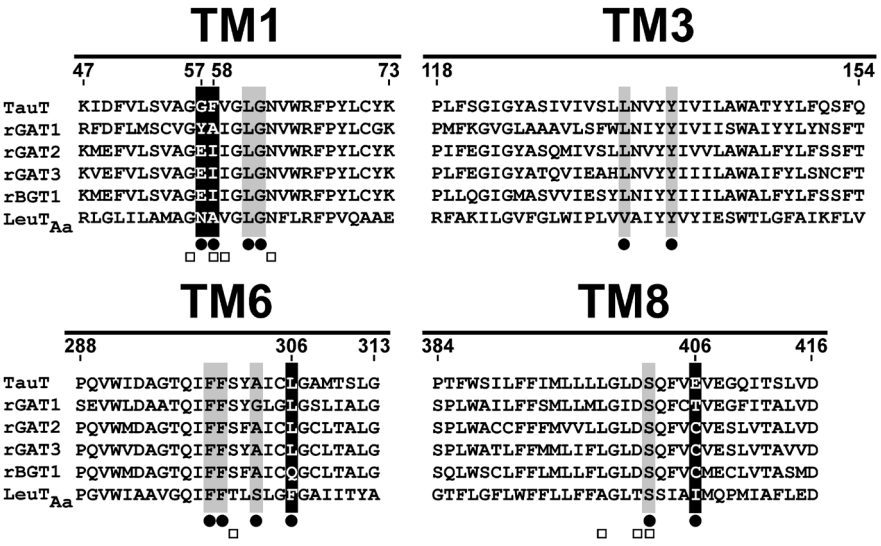

Fig. 1. Multiple Alignment of TauT, GATs and $LeuT_{Aa}$. TauT (NP_058902.1), rGAT1 (NP_077347.1), rGAT2 (NP_598307.1), rGAT3 (NP_077348.1) and rBGT1 (NP_059031.1) Were Aligned with TM Domains 1, 3, 6 and 8 of *A. aeolicus* LeuT ($LeuT_{Aa}$) (NP_214423.1)

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.

Tween-20, and incubated with 10% goat serum (Nichirei, Tokyo, Japan) for 1 h at room temperature. Immunohistochemistry was performed as described previously,[28,29] 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

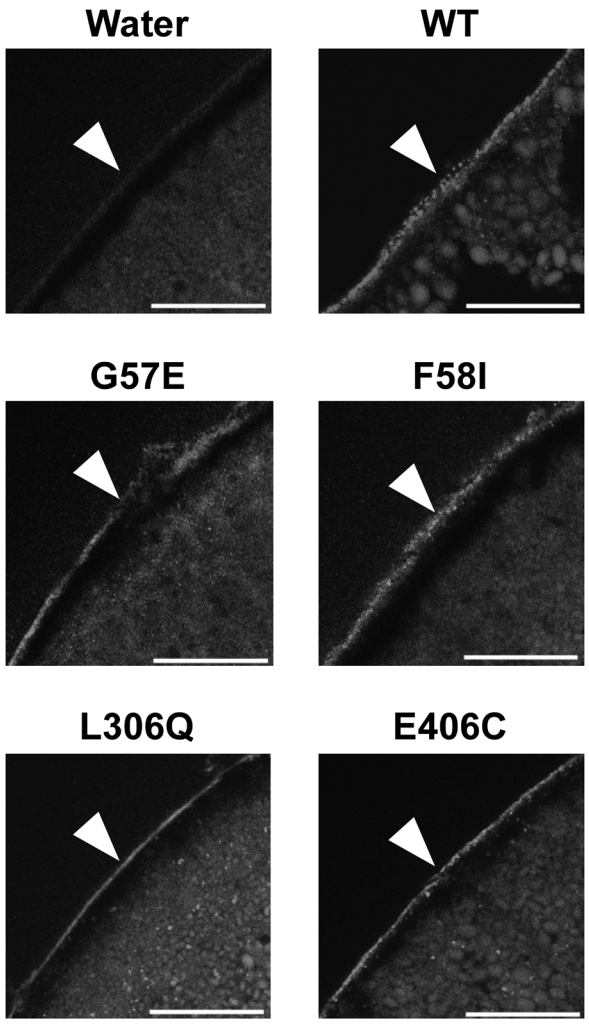

Fig. 2. Expression of TauT Proteins in Oocytes

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

G57E-expressing oocytes exhibited a marked reduction in [^{3}H]taurine and [^{3}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}H]taurine uptake by 58.1%, 97.3% and 49.0%, respectively (Fig. 4A). The uptake of [^{3}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 μM, and V_{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_{max}/K_m) for taurine were estimated to be 7.68, 4.21, 0.127 and 2.22 μL/(h·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 μM, and V_{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

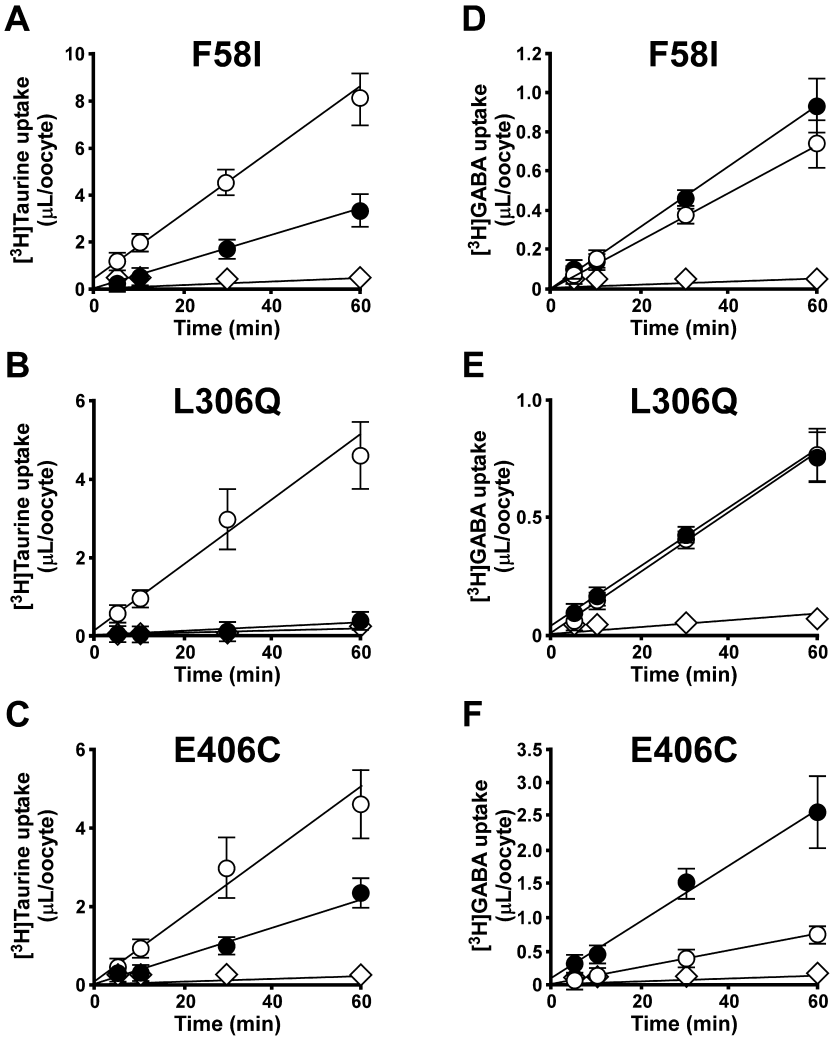

Fig. 3. Time–Course Uptake of [^{3}H]Taurine and [^{3}H]GABA by TauT-Expressing Oocytes

The uptake studies were performed for WT and TauT-mutants. The uptake of [^{3}H]taurine (125 nM) (A–C) and [^{3}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).

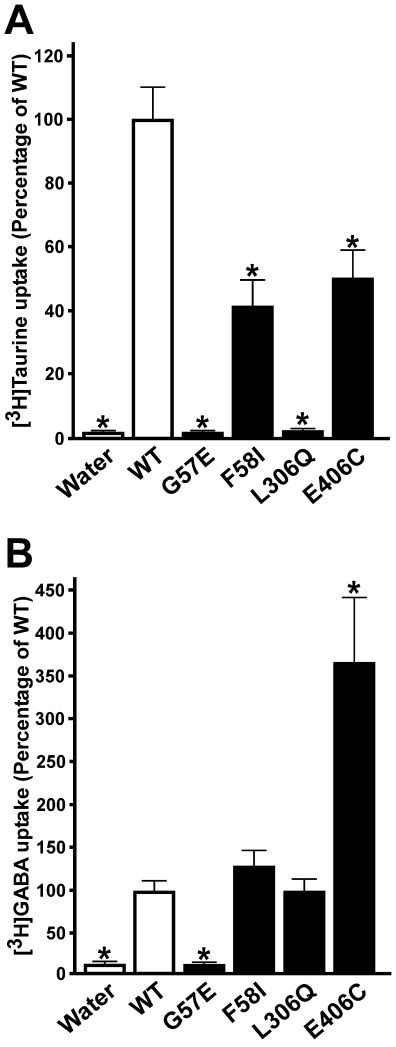

Fig. 4. Uptake of [^{3}H]Taurine and [^{3}H]GABA by TauT-Expressing Oocytes

Uptake of [^{3}H]taurine (125 nM) (A) and [^{3}H]GABA (28 nM) (B) was examined at 20°C for 60 min. Each column represents the mean±S.E.M. (n=14–73). *p<0.01, significantly different from the control.

(V_{max}/K_m) for GABA were estimated to be 0.599, 0.749, 0.365 and 2.15 μL/(h·oocyte), showing a marked increase in the uptake clearance caused by the Glu-to-Cys substitution at position 406 (Table 2).

Inhibition of Uptake by E406C-Expressing Oocytes To investigate the substrate specificity of E406C, an inhibition study was performed since E406C-expressing oocytes mostly decreased and increased the uptake clearances of taurine and GABA, respectively, compared to WT-expressing oocytes. In the case of the WT-expressing oocytes, taurine (50 μM), β-alanine (50 μM), GABA (1 mM) and nipecotic acid (2 mM) significantly inhibited [^{3}H]taurine uptake by 61.7%, 38.3%, 67.6% and 34.7%, respectively, in spite of no significant effects being caused by betaine (2 mM) and guanidinoacetic acid (GAA; 2 mM) (Table 3). In the case of the E406C-expressing oocytes, GABA and nipecotic acid significantly inhibited [^{3}H]taurine uptake by 94.9% and 96.4%, respectively, in spite of no significant effects being caused by taurine, β-alanine, betaine and GAA (Table 3). In the case of the WT-expressing oocytes, taurine (10 μM), β-alanine (10 μM), GABA (500 μM), nicopetic acid (500 μM) and GAA (2 mM) significantly inhibited [^{3}H]GABA uptake by 59.7%, 44.9%, 56.5%, 56.2% and 63.8%, respectively, in spite of no significant effects being caused by betaine (2 mM) (Table 4). In E406C-expressing oocytes, GABA, nipecotic acid and GAA significantly inhibited [^{3}H]GABA uptake by 72.6%, 86.2% and 54.9%, respectively, in spite of no significant effects being caused by taurine, β-alanine and betaine (Table 4). GAA exhibited the insignificant effect on [^{3}H]taurine uptake by TauT while it significantly inhibited the uptake of [^{3}H]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,[7,14,36] 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.[14,22] The study of $LeuT_{Aa}$, a bacterial homologue of SLC6A, 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 $LeuT_{Aa}$ (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 [^{3}H]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 [^{3}H]-taurine by G57E-, F58I-, L306Q- and E406C-expressing oocytes (Figs. 3, 4). The study of [^{3}H]GABA uptake suggested the contribution of Gly57 and Glu406 to GABA transport by TauT since G57E- and E406C-expressing oocytes exhibited significant changes in [^{3}H]GABA uptake (Figs. 3, 4).

G57E lost the activity of [^{3}H]taurine and [^{3}H]GABA uptake (Fig. 4), suggesting the role of Gly57 in the uptake function of TauT. It has been reported that TM1 of $LeuT_{Aa}$ 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 $LeuT_{Aa}$.[26,27] Therefore, regarding G57E of TauT, the reduced uptakes were caused by the Gly-to-Glu substitution that introduced a bulky side chain to the substrate pocket of TauT, suggesting the involvement of Gly57, putatively located in TM1, in providing an appropriate volume of the substrate pocket and in forming a hydrogen bond between the main chain oxygen atom and interacting with the amino group of taurine and the guanidino group of GABA.

The multiple alignments suggest that Phe58 corresponds to Ala22 of $LeuT_{Aa}$ (Fig. 1), the main chain oxygen of which

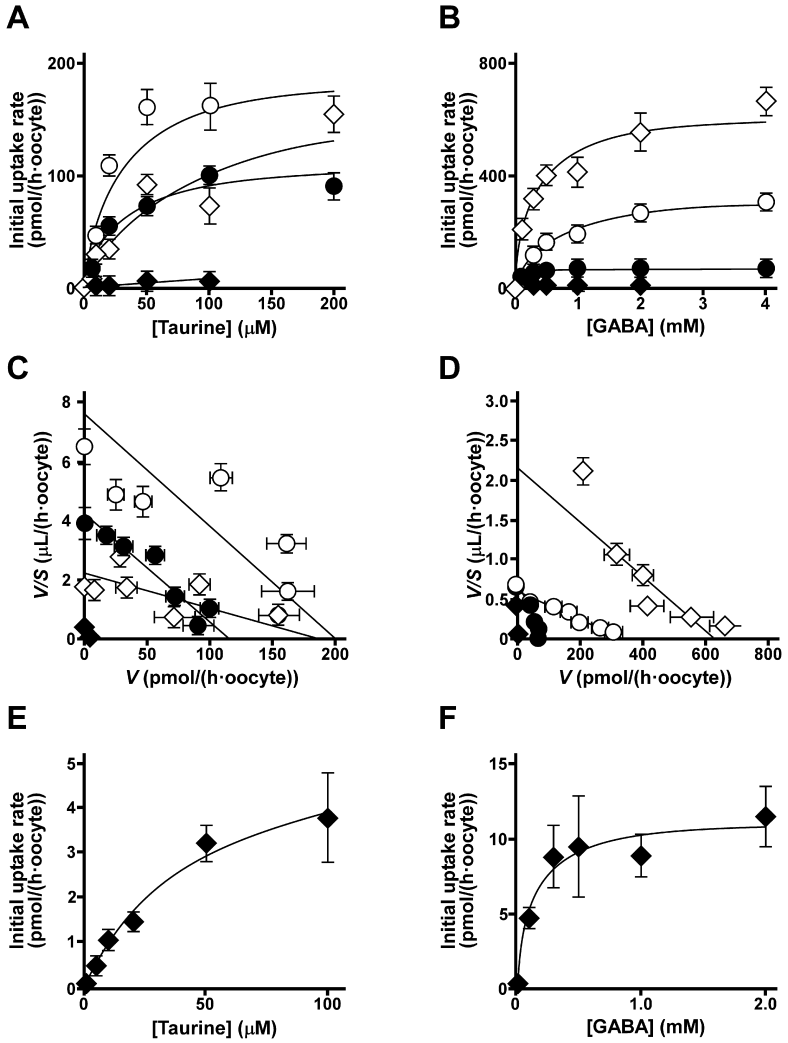

Fig. 5. Concentration-Dependent Uptake of Taurine and GABA

The uptake of taurine (A) and GABA (B), over the concentration range 5–200 μM and 0.1–4 mM, were analyzed by Michaelis–Menten kinetics, respectively. An Eadie–Scatchard plot was performed for taurine (C) and GABA (D). The uptake study with TauT-expressing oocytes was performed at 20°C for 60 min, and TauT-specific uptake was obtained by subtracting the transport rate in water-injected oocytes from that in cRNA-injected oocytes. Each symbol represents wild type (open circle), F58I (closed circle), L306Q (closed diamond) and E406C (open diamond), respectively. The magnified plots for the uptake of taurine and GABA by L306Q-expressing oocytes were shown in panel E and F, respectively. Each point represents the mean±S.E.M. (n=11–30).

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

Mutants	Taurine			GABA		
	K_m (μM)	V_{max} (pmol/(h·oocyte))	V_{max}/K_m (μL/(h·oocyte))	K_m (μM)	V_{max} (pmol/(h·oocyte))	V_{max}/K_m (μL/(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.

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 $LeuT_{Aa}$, 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 μM) for GABA than that of WT (K_m=564 μM) (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 $LeuT_{Aa}$ has been reported to

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

Inhibitors	Concentration (μM)	Relative uptake (% of control)	
		Wild-type	E406C
Control		100±8	100±15
Taurine	50	38.3±3.7*	62.7±6.3
β-Alanine	50	61.7±5.6*	153±28
GABA	1000	32.4±6.3*	5.08±0.86*
Nipecotic 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}H]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, γ-aminobutyric acid; GAA, guanidinoacetic acid.

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

Inhibitors	Concentration (μM)	Relative uptake (% of control)	
		Wild-type	E406C
Control		100±11	100±11
Taurine	10	40.3±9.2*	104±13
β-Alanine	10	55.1±13.2**	113±15
GABA	500	43.5±5.7*	27.4±3.9*
Nipecotic 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}H]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, γ-aminobutyric acid; GAA, guanidinoacetic acid.

be a residue forming the Na^+-binding site that is essential for the function of $LeuT_{Aa}$,[24,37] and the insignificant change in [^{3}H]GABA uptake by F58I strongly suggests a minor contribution of Phe58 in the Na^+-binding of TauT (Figs. 3D, 4B).

In TM6 of $LeuT_{Aa}$, the side chain of Phe259 has been reported to be involved in a hydrophobic interaction with the isopropyl-group of L-leucine,[24,29] and the multiple alignments suggest that Phe259 of $LeuT_{Aa}$ 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}H]taurine uptake by 97% while there was no significant effect on [^{3}H]GABA uptake (Fig. 4). This is also supported by the transport clearance (V_{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 μM) and GABA (K_m=22.6 μM) 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 $LeuT_{Aa}$ (Fig. 1), and the side chain of Ile359 in $LeuT_{Aa}$ has been reported to form a hydrophobic interaction with L-leucine,[24] implying the involvement of Glu406 in the substrate recognition of TauT. The uptake study suggests the contribution of Glu406 to the [^{3}H]taurine and [^{3}H]GABA transport by TauT since E406C exhibited significantly lower and greater uptake of [^{3}H]taurine and [^{3}H]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_{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, Fig. 5). According to the previous reports, the polar side chain of Glu406 was suggested to make only a minor contribution to the substrate recognition of TauT since the polar side chain of Thr400 in hGAT1, corresponding to Glu406 of TauT, has been reported to play only a minor role in the function of hGAT1.[38,39] Thus, the higher affinity for GABA supports the involvement of Glu406 in determining the volume of the substrate pocket since it is thought that the Glu-to-Cys substitution at position 406 reduced the side chain bulkiness to improve the accessibility of GABA to the substrate pocket. These findings support the hypothesis that the lower affinity of E406C for taurine was caused by an increased volume of the substrate pocket, that weakened the interaction between taurine and the putative TM8 of TauT.

Furthermore, E406C exhibited the highest uptake activity and transport clearance (V_{max}/K_m) for GABA (Table 2), implying that Glu406 is the most important residue among the four residues examined here. The inhibitory effect study revealed differences in transport between E406C and WT. Regarding [^{3}H]taurine transport, E406C showed its GATs-like transport property since the Glu-to-Cys substitution exhibited a lower

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 [^{3}H]GABA by E406C (Table 4). Although amino acid substitution was performed by referring the sequence of rBGT1, 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 rBGT1, 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 recognition ability 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.

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