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Function of taurine transporter (Slc6a6/TauT) as a GABA transporting protein and its relevance to GABA transport in rat retinal capillary endothelial cells

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ABSTRACT

The purpose of this study was to identify the uptake mechanism of γ-aminobutyric acid (GABA) via taurine transporter (Slc6a6/TauT) and its relationship with GABA transport at the inner BRB. Rat Slc6a6/TauT-transfected HeLa cells exhibited Na^+-, Cl^--, and concentration-dependent [^{3}H]GABA uptake with a K_m of 1.5 mM. Taurine, β-alanine, and GABA markedly inhibited Slc6a6/TauT-mediated uptake of [^{3}H]GABA. The uptake of [^{3}H]GABA by a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2) was Na^+-, Cl^--, and concentration-dependent with a K_m of 2.0 mM. This process was more potently inhibited by substrates of Slc6a6/TauT, taurine and β-alanine, than those of GABA transporters, GABA and betaine. In the presence of taurine, there was competitive inhibition with a K_i of 74 μM. [^{3}H]Taurine also exhibited competitive inhibition with a K_i of 1.8 mM in the presence of GABA. In conclusion, rat Slc6a6/TauT has the ability to use GABA as a substrate and Slc6a6/TauT-mediated GABA transport appears to be present at the inner BRB.

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1. Introduction

Retinal γ-aminobutyric acid (GABA) plays a key role as an inhibitory neurotransmitter [1]. The neurotransmitter actions of GABA in the synaptic cleft are terminated by its reuptake by either pre-synaptic neurons or nearby glial cells via GABA transporters [2]. Four GABA transporters have been isolated and all of them belong to the family of Na^+- and Cl^--dependent neurotransmitter transporters: Slc6 [3]. Slc6a1, Slc6a11, Slc6a12, and Slc6a13, respectively, correspond to GABA transporter (GAT)-1, GAT-3, betaine/GABA transporter (BGT)-1, and GAT-2 in rats and GAT1, GAT4, GAT2, and GAT3 in mice.

The inner blood-retinal barrier (BRB) forms complex tight junctions of retinal capillary endothelial cells to restrict non-specific transport between the circulating blood and neural retina [4,5]. Transporters at the inner BRB are thought to play essential roles in supplying nutrients and be also responsible for the efflux of neurotransmitters and their metabolites from the retina to the circulating blood for the maintenance of neural functions. Recently, we reported that a member of the Slc6 transporter family, taurine transporter (Slc6a6/TauT), is expressed in retinal capillary endothelial cells and mediates the transport of taurine [6]. Although it has been reported that transfection with rat Slc6a6/TauT in COS cells did not enhance the uptake of [^{3}H]GABA [7], rat Slc6a6/TauT is most closely related to rat Slc6a11/GAT-3 and Slc6a13/GAT-2 (61% amino acid identity) and it has been referred to an "honorary GABA transporter" (for a review, see [8]). Consequently, Slc6a6/TauT at the inner BRB might play an important role in reducing the GABA concentration in the retinal interstitial fluid.

The purpose of the present study was to clarify whether rat Slc6a6/TauT recognizes GABA as a substrate using Slc6a6/TauT-overexpressing cells. Furthermore, we investigated the GABA transport properties at the inner BRB using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2) [5,9].

2. Materials and methods

2.1. Cell culture

TR-iBRB2 cells possess endothelial markers and facilitative glucose transporter 1 (GLUT1), P-glycoprotein, creatine transporter (Slc6a8/CRT), and L-type amino acid transporter 1 (LAT1) which are expressed at the inner BRB in vivo [9–11]. Thus, TR-iBRB2 cells maintain certain in vivo functions and are a suitable in vitro model for the inner BRB. Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, and 10% fetal bovine serum (FBS; Moregate, Bulimbra, Australia) was used as the culture medium for TR-iBRB2 cells. TR-iBRB2 cells were seeded onto rat tail collagen type I-coated tissue culture plates (BD Biosciences, Bedford, MA, USA) and cultured at 33 °C in a humidified atmosphere of 5% CO_2/air. The permissive-temperature for TR-iBRB2 cell culture is 33 °C due to the presence of temperature-sensitive SV 40 large T-antigen [9]. HeLa cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2/air. The culture medium for HeLa cells consisted of DMEM supplemented with 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, 25 mM D-glucose, and 10% FBS.

2.2. cDNA isolation and expression of rat taurine transporter (Slc6a6/TauT)

Total cellular RNA was prepared from TR-iBRB2 cells using an Rneasy Kit (Qiagen, Hilden, Germany). Single-strand cDNA was made from total RNA by reverse transcription (RT) using oligo dT primer. The polymerase chain reaction (PCR) was performed with rat Slc6a6/TauT (GenBank accession number NM_017206) specific primers through 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min. The sequences of specific

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primers of rat Slc6a6/TauT were as follows: the sense primer with a BamHI site (underlined) was 5′-GGA TCC ATG GCC ACC AAG GAG AAG CTT CA-3′ and the antisense primer with an XhoI site (underlined) was 5′-CTC GAG TCA CAT CAT GGT CTC CAC AAT GAC-3′. The PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide and visualized under ultraviolet light. The PCR product at 1878 bp was then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and amplified in Escherichia coli. Several clones were then sequenced from both directions using a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA, USA). The cDNA segment 100% identical to the full-length of rat Slc6a6/TauT was cut from pGEM-T Easy Vector using BamHI and XhoI (Roche, Mannheim, Germany), and subcloned into pcDNA4/HisC vector (Invitrogen, Carlsbad, CA, USA), which contains Xpress epitope in the upstream of the multiple cloning site.

Constructed pcDNA4/HisC/TauT or pcDNA4/HisC vector alone (termed mock) was transfected into HeLa cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, HeLa cells were seeded at a density of 4×10^4 cells/cm^2 on a 24-well tissue culture plate (BD Biosciences) or Lab-Tek chamber 8-well slide (Nalge Nunc, Rochester, NY, USA). At 1 day after seeding, the cells were exposed to antibiotic-free DMEM containing 25 mM D-glucose and 10% FBS and then transfected with complexes of 1.0 μg/10^5 cells of pcDNA4/HisC/TauT or pcDNA4/HisC and 1.9 μL/10^5 cells of Lipofectamine 2000 reagent in OPTI-MEMI medium (Invitrogen). At 6 h after the initiation of transfection, the medium was changed to antibiotic-free DMEM containing 25 mM D-glucose and 10% FBS.

2.3. Immunohistochemical analysis

pcDNA4/HisC/TauT or mock-transfected HeLa cells were cultured on Lab-Tek chamber 8-well slides and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. Cells were incubated with methanol containing 5% acetic acid and 5% water for 10 min at −20 °C and then incubated with 10% goat serum (Nichirei, Tokyo, Japan) for 30 min at room temperature. Cells were then incubated with mouse monoclonal anti-Xpress antibody (1:240; Invitrogen) for 16 h at 4 °C and subsequently incubated with FITC-conjugated anti-mouse IgG antibody (1:200; Chemicon, Temecula, CA, USA) for 2 h at room temperature. Photographs were taken with a confocal laser scanning microscope (LSM 510; Carl Zeiss Meditec, Oberkochen, Germany).

2.4. [^{3}H]GABA and [^{3}H]taurine uptake

The 4-amino-n-[2,3-^{3}H]butyric acid ([^{3}H]GABA, 93.0 Ci/mmol, GE Healthcare Bio-Sciences, Buckinghamshire, UK) or [2-^{3}H(N)]taurine ([^{3}H]taurine, 30.3 Ci/mmol, Perki-nElmer Life Science, Boston, MA, USA) uptake was measured according to a previous report [6]. Briefly, TR-iBRB2 cells and pcDNA4/HisC/TauT or mock-transfected HeLa cells were cultured on 24-well plates (BD Biosciences) and washed with 1 mL extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO$_3$, 3 mM KCl, 1.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, 10 mM D-glucose, and 10 mM Hepes (pH 7.4) at 37 °C. Uptake was initiated by applying 200 μL ECF buffer containing 0.2 μCi [^{3}H]GABA (11 nM) or 0.1 μCi [^{3}H]taurine (17 nM) at 37 °C in the presence or absence of inhibitors. Na$^+$- or Cl$^−$-free ECF buffer were obtained by replacement with equimolar choline or gluconate, respectively. After a predetermined period, uptake was terminated by removing the solution, and cells were washed with 1 mL ice-cold ECF buffer. The cells were then solubilized in 1 N NaOH and subsequently neutralized. An aliquot was taken for measurement of radioactivity and protein content using, respectively, a liquid scintillation counter and a DC protein assay kit (Bio-rad, Hercules, CA, USA) with bovine serum albumin as a standard.

For kinetic studies, the Michaelis–Menten constant (K_m) and maximum rate (J_{max}) of GABA or taurine uptake were calculated from Eq. (1) using the nonlinear least-square regression analysis program, MULTI [12].

$$J = J_{max} \times C/(K_m + C) \tag{1}$$

where J and C are the uptake rate of GABA or taurine and the concentration of GABA or taurine, respectively. To analyze the mechanism of inhibition of [^{3}H]GABA uptake by taurine and [^{3}H]taurine uptake by GABA, the inhibitory constant (K_i) was calculated from Eq. (2) using MULTI [12].

$$J = J_{max} \times C/[K_m \times (1 + I/K_i) + C] \tag{2}$$

where I is the concentration of GABA or taurine.

2.5. Data analysis

Unless otherwise indicated, all data represent means±S.E.M. An unpaired, two-tailed Student's t-test was used to determine the significance of differences between two groups. Statistical significance of differences among means of several groups was determined by one-way analysis of variance followed by the modified Fisher's least-squares difference method.

3. Results

3.1. GABA uptake mediated by Slc6a6/TauT

Fig. 1A shows the confocal microscopic images of Xpress-tagged Slc6a6/TauT-transfected HeLa cells stained with anti-Xpress antibody.
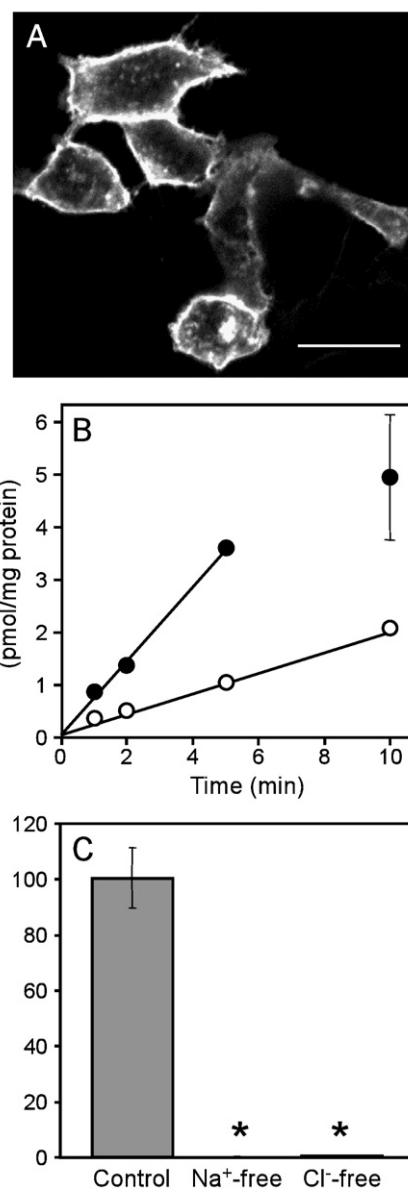

Fig. 1. Localization (A) and function (B and C) of Xpress-tagged rat Slc6a6/TauT in transfected HeLa cells. (A) Immunostaining of Xpress-tagged rat Slc6a6/TauT-trans-fected HeLa cells. Anti-Xpress antibody was used for immunostaining. Scale bar, 20 μm. (B) Time-course of [^{3}H]taurine (17 nM) uptake at 37 °C by rat Slc6a6/TauT-transfected (closed circle) and mock-transfected (open circle) cells. (C) Na$^+$- and Cl$^−$-dependence of [^{3}H]taurine uptake by rat Slc6a6/TauT-transfected cells. [^{3}H]Taurine (17 nM) uptake by rat Slc6a6/TauT-transfected cells was performed in the presence (control) or absence of Na$^+$ and Cl$^−$ at 37 °C for 5 min. Each point represents the mean±S.E.M. (n=4). *p<0.01 significantly different from control.

Intense immunoreactivity was detected at the plasma membrane in Slc6a6/TauT-transfected cells, whereas no signal was seen in the mock-transfected cells (data not shown). The initial uptake rate of [^{3}H] taurine in Slc6a6/TauT-transfected cells was 3.6-fold greater than that in mock-transfected cells (Fig. 1B). Moreover, the absence of either Na$^+$ or Cl$^−$ reduced the Slc6a6/TauT-mediated uptake of [^{3}H]taurine by more than 90% (Fig. 1C). These results indicate that the introduced Slc6a6/TauT acts at the plasma membrane in Slc6a6/TauT-transfected cells.

The time-courses of [^{3}H]GABA in Slc6a6/TauT- and mock-trans-fected cells are shown in Fig. 2A. The initial uptake rate of [^{3}H]GABA in Slc6a6/TauT-transfected cells was 52.5 fmol/(min·mg protein) and 4.7-fold greater than that in mock-transfected cells (11.2 fmol/(min·mg
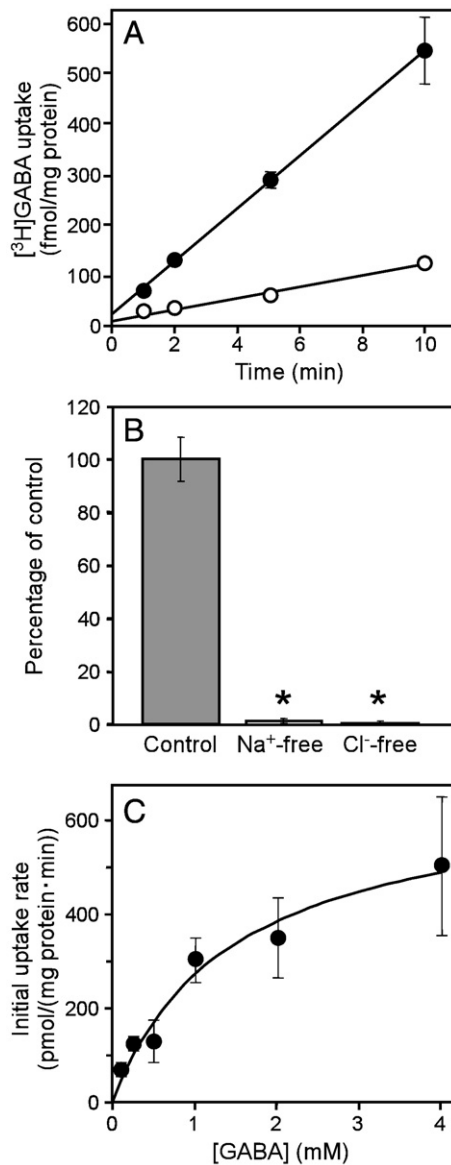
Fig. 2. Time-course (A), Na$^+$ and Cl$^-$-dependence (B), and concentration-dependence (C) of [^{3}H]GABA uptake by rat Slc6a6/TauT-transfected cells. (A) Time-course of [^{3}H]GABA (11 nM) uptake at 37 °C by rat Slc6a6/TauT-transfected (closed circle) and mock-transfected (open circle) cells. (B) [^{3}H]GABA (11 nM) uptake was performed in the presence (control) or absence of Na$^+$ and Cl$^-$ at 37 °C for 10 min. (C) [^{3}H]GABA (11 nM) uptake was performed at 37 °C for 10 min. K_m is 1.46±0.47 mM and J_{max} is 666± 93 pmol/(min·mg protein) (mean±S.D.) according to Eq. (1). Each point represents the mean±S.E.M. ($n=4$). *$p<0.01$ significantly different from control.

protein), indicating that rat Slc6a6/TauT recognizes GABA as a substrate as well as taurine. The Slc6a6/TauT-mediated uptake of GABA took place in a concentration-dependent manner (Fig. 2C). Nonlinear, least-squares regression analysis was performed to give the best fit using the uptake rate for 10 min, yielding the following kinetic

Table 1
K_m values for the uptake of GABA via Slc6 transporters

Transporters	K_m	Species	Reference
Slc6a1/GAT-1	7.0 μM	Rat	[13]
Slc6a6/TauT	1.5 mM	Rat	Fig. 2C
Slc6a11/GAT-3	2.3–12 μM	Rat	[14,15]
Slc6a12/GAT2	79 μM	Mouse	[16]
Slc6a13/GAT-2	8 μM	Rat	[15]

Table 2
Effect of several inhibitors on TauT-mediated [^{3}H]GABA uptake by TauT-transfected HeLa cells

Inhibitors	Percentage of control
Control	100±3
2 mM taurine	3.80±1.31*
2 mM β-alanine	4.30±0.06*
2 mM γ-aminobutyric acid	28.7±4.5*
2 mM L-leucine	80.9±16.1
2 mM L-α-alanine	93.0±4.8

[^{3}H]GABA uptake (11 nM) was performed in the absence (control) or presence of inhibitors at 37 °C for 10 min. Each value represents the mean±S.E.M. ($n=4$–12). *$p<0.01$, significantly different from the control.

parameters: a K_m and J_{max} of 1.46±0.47 mM and 666±93 pmol/ (min·mg protein) (mean±S.D.), respectively. This K_m value is much higher than those in other GABA transporters [13–16] (Table 1).
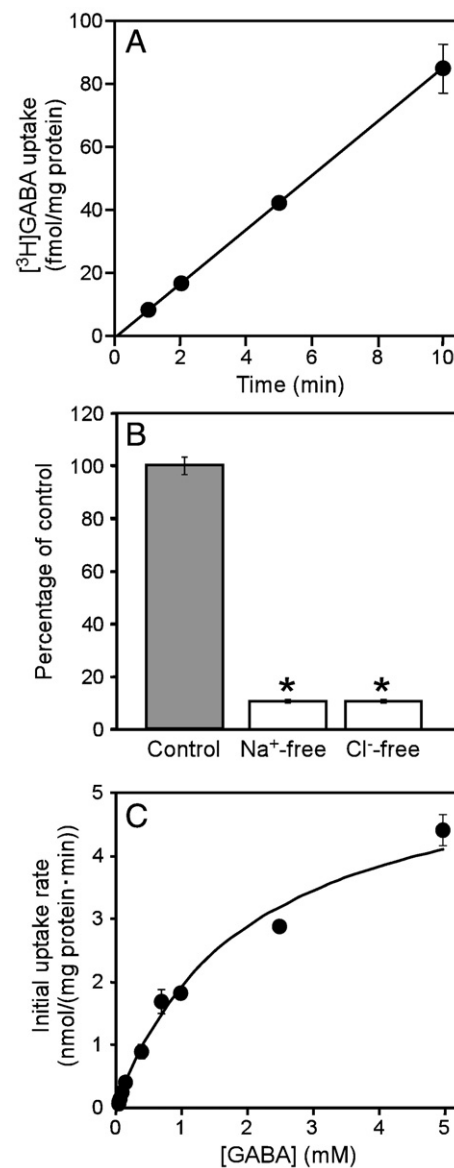

Fig. 3. Time-course (A), Na$^+$- and Cl$^-$-dependence (B), and concentration-dependence (C) of [^{3}H]GABA uptake by TR-iBRB2 cells. (B) [^{3}H]GABA (11 nM) uptake was performed in the presence (control) or absence of Na$^+$ and Cl$^-$ at 37 °C for 10 min. (C) [^{3}H]GABA (11 nM) uptake was performed at 37 °C for 10 min. K_m is 2.04±0.33 mM and J_{max} is 5.80± 0.53 nmol/(min·mg protein) (mean±S.D.) according to Eq. (1). Each point represents the mean±S.E.M. ($n=4$). *$p<0.01$ significantly different from control.

Table 3
Effect of several inhibitors on [³H]GABA uptake by TR-iBRB2 cells

Inhibitors	Percentage of control
Control	100±1
2 mM taurine	14.3±1.3*
2 mM β-alanine	16.4±2.1*
2 mM γ-aminobutyric acid	38.9±1.9*
500 μM nipecotic acid	83.2±4.5*
2 mM nipecotic acid	45.8±2.6*
2 mM betaine	86.3±3.2*
2 mM ʟ-leucine	85.8±5.2*
2 mM ʟ-α-alanine	70.5±2.8*
2 mM creatine	75.6±3.8*
2 mM probenecid	96.4±3.9

[³H]GABA uptake (11 nM) was performed in the absence (control) or presence of inhibitors at 37 °C for 10 min. Each value represents the mean±S.E.M. (n=4–12). *p<0.01, significantly different from the control.

To characterize Slc6a6/TauT-mediated uptake of [³H]GABA, we examined the effects of several inhibitors of Slc6a6/TauT and other compounds on Slc6a6/TauT-mediated uptake of [³H]GABA (Table 2). Taurine, β-alanine, and GABA at a concentration of 2 mM markedly inhibited Slc6a6/TauT-mediated uptake of [³H]GABA by more than 70%. In contrast, ʟ-leucine and ʟ-alanine had no significant effect on Slc6a6/TauT-mediated uptake of [³H]GABA. Moreover, the absence of either Na⁺ or Cl⁻ reduced the Slc6a6/TauT-mediated uptake of [³H]GABA by more than 90% (Fig. 2B). These characteristics of Slc6a6/TauT-mediated uptake of GABA are consistent with those of taurine [7,17].

3.2. GABA transport system in TR-iBRB2 cells

[³H]GABA uptake was performed using TR-iBRB2 cells as an in vitro model of the inner BRB in order to determine the kinetic parameters of GABA and characterize GABA transport at the inner BRB. The time-course of [³H]GABA uptake by TR-iBRB2 cells is shown in Fig. 3A. [³H]GABA uptake increased linearly for 10 min and the initial uptake rate was 8.45 fmol/(min·mg protein). In contrast, [³H]GABA uptake was reduced by 89.6% and 89.5% in the absence of either Na⁺ or Cl⁻, respectively (Fig. 3B), suggesting that GABA uptake by TR-iBRB2 cells takes place in an Na⁺-, and Cl⁻-dependent manner.

The effect of amino acids and an organic anion on [³H]GABA uptake by TR-iBRB2 cells is summarized in Table 3. Taurine and β-alanine at a concentration of 2 mM markedly inhibited [³H]GABA uptake by more than 80%. GABA at a concentration of 2 mM inhibited [³H]GABA uptake by 61%. Nipecotic acid inhibited [³H]GABA uptake by 17% and 54% at a concentration of 500 μM and 2 mM, respectively. Betaine, ʟ-leucine, ʟ-alanine, and creatine at a concentration of 2 mM had a weaker effect and 2 mM probenecid had no significant effect on [³H]GABA uptake. The uptake of GABA by TR-iBRB2 cells was concentration-dependent with a K_m and J_{max} of 2.04±0.33 mM and 5.80±0.53 nmol/(min·mg protein) (mean±S.D.), respectively (Fig. 3C). Moreover, the Eadie–Scatchard plot showed that the two lines of the GABA uptake in the presence or absence of 50 μM taurine intersected on the abscissa. This indicates that taurine competitively inhibited GABA uptake with a K_i of 73.8±8.8 μM (mean±S.D.) (Fig. 4A). In the presence of 5 mM of GABA, [³H]taurine uptake by TR-iBRB2 cells also exhibited competitive inhibition with a K_i of 1.80±0.01 mM (mean±S.D.) (Fig. 4B). These results confirm the presence of Slc6a6/TauT-mediated uptake of GABA in TR-iBRB2 cells.

4. Discussion

The present study demonstrates that rat Slc6a6/TauT-transfected HeLa cells exhibit Na⁺, Cl⁻, and concentration-dependent GABA uptake (Fig. 2). It has been reported that transfection with rat Slc6a6/TauT into COS cells did not result in enhanced uptake of [³H]GABA [7]. These controversial observations suggest that Slc6a6/TauT-mediated GABA transport activity depends on the cell type and preparation used.

The crystal structure of a bacterial homolog of Slc6 transporter from *Aquifex aeolicus* (leucine transporter; LeuT$_{Aa}$) predicts that 12 residues in transmembrane segments 1, 3, 6, and 8 are involved in substrate binding in Slc6 transporters [18]. Creatine transporter (Slc6a8/CRT) is reported to lose creatine transport activity and gain a GABA transport function by combinations of 2 or 3 amino acid substitutions at the 12 residues involved in substrate binding with their counterpart in Slc6a1/GAT-1 [19]. The amino acid sequence alignment of rat Slc6a6/TauT, Slc6a11/GAT-3, Slc6a12/BGT-1, and Slc6a13/GAT-2 at the 12 residues involved in substrate binding showed that only 3 of the 12 residues in Slc6a6/TauT are different from those in Slc6a11/GAT-3, Slc6a12/BGT-1, and Slc6a13/GAT-2, implying that the substrate binding sites of Slc6a6/TauT are capable of recognizing GABA as a substrate. On the other hand, the substitution of glutamate-61 of mouse Slc6a11/GAT4, one of the 3 amino acid differences in the 12 residues involved in substrate binding, with its Slc6a6/TauT counterpart glycine markedly increased the inhibitory potency of taurine on the uptake of [³H]GABA [20]. This evidence suggests that the 3 amino acid differences in the 12 residues involved in substrate binding play a key role in high-affinity transport of taurine in Slc6a6/TauT.

We have recently reported that Slc6a6/TauT is expressed and predominantly involved in taurine transport in rat retinal capillary endothelial cells (TR-iBRB2) [6]. The uptake of [³H]GABA by TR-iBRB2 cells took place in an Na⁺-, Cl⁻-, and concentration-dependent manner (Fig. 3). The corresponding K_m of 2.0 mM (Fig. 3C) is in good agreement with that obtained for GABA uptake by rat Slc6a6/TauT expressed HeLa cells (K_m=1.5 mM) (Fig. 2C) but is much higher than those in other GABA transporters [13–16] (Table 1). Typical Slc6a6/TauT substrates, such as
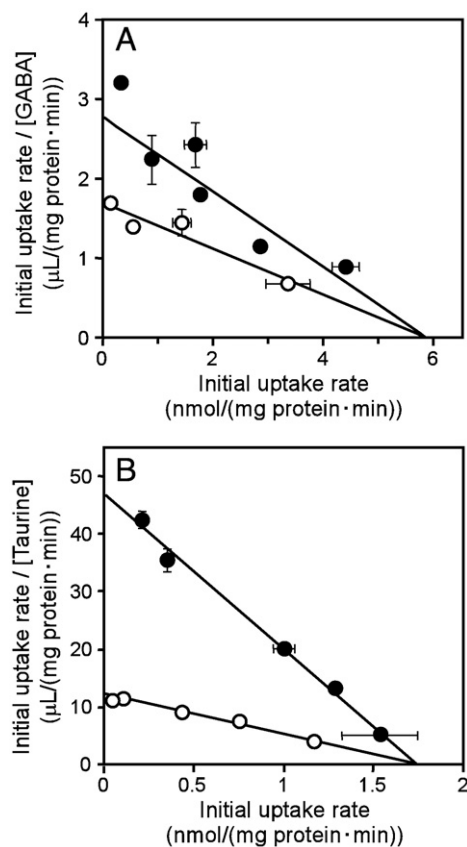

Fig. 4. Eadie–Scatchard plot of GABA (A) and taurine (B) uptake by TR-iBRB2 cells showing mutual competitive inhibition of the uptake of taurine and GABA. (A) [³H]GABA uptake was performed in the absence (closed circle) or presence of 50 μM taurine (open circle) at 37 °C for 10 min. The K_i for taurine is 73.8±8.8 μM (mean±S.D.) according to Eq. (2). (B) [³H]Taurine uptake was performed in the absence (closed circle) or presence of 5 mM GABA (open circle) at 37 °C for 5 min. The K_i for GABA is 1.80±0.01 mM (mean±S.D.) according to Eq. (2). Each point represents the mean±S.E.M. (n=4).

taurine and β-alanine, strongly inhibited [³H]GABA uptake by TR-iBRB2 cells (Table 3). These forms of inhibition are consistent with those obtained for [³H]GABA uptake by rat Slc6a6/TauT expressed HeLa cells (Table 2). Inhibitory effect of nipecotic acid on [³H]GABA uptake by TR-iBRB2 cells (Table 3) was much less than those observed in mouse Slc6a1/GAT1, Slc6a11/GAT4, and Slc6a13/GAT3 [21]. Betaine, a specific substrate of Slc6a12/BGT-1 [16,21], produced no marked inhibition of this process (Table 3). Furthermore, mutual inhibition was observed for GABA and taurine uptake by TR-iBRB2 cells. The [³H]GABA uptake was competitively inhibited by 50 μM taurine with a K_i of 74 μM (Fig. 4A), which is comparable with the K_m of 22 μM for taurine uptake by TR-iBRB2 cells [6]. The [³H]taurine uptake was also competitively inhibited by 5 mM GABA with a K_i of 1.8 mM (Fig. 4B), which is comparable with the K_m of 2.0 mM for GABA uptake by TR-iBRB2 cells (Fig. 3C). These results suggest that Slc6a6/TauT plays a key role in the transport of GABA as well as taurine at the inner BRB. Although the physiological relevance of Slc6a6/TauT as a GABA transporter is not revealed as extracellular concentrations of GABA and taurine in the retina are unknown, the low-affinity GABA transport via Slc6a6/TauT might make a greater contribution to the removal of GABA from retinal interstitial fluid when GABA is released from the intracellular spaces under hyposmosis and ischemia [22].

In conclusion, the data presented here provides the first evidence that GABA is actually a substrate of Slc6a6/TauT. Slc6a6/TauT appears to play a key role in transporting GABA at the inner BRB. These findings provide important information to increase our understanding of the substrate binding mechanisms of Slc6 transporters and the physiological roles of GABA transporters in neural tissues.

Acknowledgements

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