Hypotaurine Is a Substrate of GABA Transporter Family Members GAT2/Slc6a13 and TAUT/Slc6a6

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Hypotaurine is a precursor of taurine and a physiological antioxidant that circulates in adult and fetal plasma. The purpose of the present study was to clarify whether hypotaurine is a substrate of Slc6a/gamma-aminobutyric acid (GABA) transporter family members. Radiolabeled hypotaurine was synthesized from radiolabeled cysteamine and 2-aminoethanethiol dioxygenase. The uptakes of [3H]GABA, [3H]taurine, and [14C]hypotaurine by HEK293 cells expressing mouse GAT1/Slc6a1, TAUT/Slc6a6, GAT3/Slc6a11, BGT1/Slc6a12, and GAT2/Slc6a13 were measured. TAUT and GAT2 showed strong [3H]hypotaurine uptake activity, while BGT1 showed moderate activity, and GAT1 and GAT3 showed slight but significant activity. Mouse TAUT and GAT2 both showed Michaelis constants of 11 μM for hypotaurine uptake. GAT2-expressing cells pretreated with hypotaurine showed resistance to H2O2-induced oxidative stress. These results suggest that, under physiological conditions, TAUT and GAT2 would be major contributors to hypotaurine transfer across the plasma membrane, and that uptake of hypotaurine via GAT2 contributes to the cellular resistance to oxidative stress.

Key words hypotaurine; antioxidant; gamma-aminobutyric acid (GABA); taurine; transporter

Hypotaurine, which is a precursor of taurine, is synthesized from cysteine via two pathways: one mediated by cysteine dioxygenase (CDO) and cysteine sulfenic acid decarboxylase, and one mediated by cysteamine dioxygenase (ADO). Hypotaurine is converted to taurine by hypotaurine deoxygenase or via non-enzymatic oxidation.1-3 The non-enzymatic oxidation of hypotaurine to taurine by reactive oxygen species (ROS) serves to scavenge ROS.1,2,4,5 Hypotaurine concentration correlates with the motility of spermatozoa, and reduces their level of ROS.6,7 Hepatic ischemia-reperfusion induces an increase of cellular hypotaurine.8 Ezrin gene (Vil2)-knockout mice showed fetal growth retardation and hypotaurine deficiency in fetal plasma.9 Thus, hypotaurine serves as an endogenous antioxidant, and proteins that regulate intracellular hypotaurine level are likely to play a role in cellular responses to oxidative stress.

Hypotaurine is enriched in several tissues, reaching millimolar concentration in rat liver regenerating after partial hepatectomy and leukocytes, and its concentration is 0.1 mM in fetal plasma of mouse and bovine.10,11 Intracellular hypotaurine can be supplied by biosynthesis via CDO and ADO and also by uptake via concentrative membrane transporters. Indeed, we have shown that intracellular hypotaurine is enriched dependently upon extracellular hypotaurine in placental trophoblasts.12

Although the enzymes involved in hypotaurine biosynthesis have been investigated, the transporters responsible for hypotaurine transport across the plasma membrane have not been identified. It has been reported that the hypotaurine transport system is Na+-dependent and resembles the gamma-aminobutyric acid (GABA) and taurine transporters in mouse brain slices, neuroblastoma, and rat retina.11-16 Further, hypotaurine inhibits the transport of β-amino acids such as GABA, β-alanine and taurine. It is well established that Slc6a transporter family members are involved in the transport of β-amino acids. Also, hypotaurine inhibits the transport activity of several Slc6a/GABA transporters.17,18 Therefore, Slc6a members may be involved in hypotaurine transport.

Thus, the purpose of the present study was to clarify whether hypotaurine is a substrate of mouse Slc6a/GABA transporter family members, Slc6a1, Slc6a6, Slc6a11, Slc6a12 and Slc6a13. In the case of Slc6a family members, the relationship of approved gene symbols and their synonymous GABA transporter names is not consistent among species. In this article, we use the GABA transporter nomenclature GAT1 for Slc6a1, TAUT for Slc6a6, GAT3 for Slc6a11, BGT1 for Slc6a12 and GAT2 for Slc6a13, as approved by the Human Genome Organisation (HUGO) gene nomenclature committee.

MATERIALS AND METHODS

Materials [1,2,14C]Cysteamine hydrochloride ([14C]-cysteamine, 55 mCi/mmol) and gamma-[2,3-3H]aminobutyric acid ([3H]GABA, 60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). [2,3-3H(N)]Taurine ([3H]taurine, 22.7 Ci/mmol) was purchased from PerkinElmer, Inc. (Boston, MA, U.S.A.). Unlabeled cysteamine and hypotaurine were purchased from Sigma (St. Louis) and taurine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). pcDNA5/FRT/TO vector, pOG44 vector, Flp-In T-REx 293 cells and mouse GAT3/Slc6a11 cDNA/pENTR223 (Invitrogen, CA, U.S.A.) were purchased from Addgene (Cambridge, MA, U.S.A.) and were a generous gift from Dr. K SowZXn, National Institute of Health, Japan. The human genome organization database (www.gene.com/).
from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Mouse GAT1/Slc6a1 cDNA/pYX-Asc (BC059080) and mouse BGT1/Slc6a12 cDNA/pCMV-SPORT6 (BC019211) were purchased from Origene Technologies (Rockville, MD, U.S.A.). Fully sequenced mouse TAUT/Slc6a6 and mouse GAT2/Slc6a13 complete cDNA/pCMV-SPORT6 (BC015245 and BC029637, respectively) were purchased from Dharmacon (Lafayette, CO, U.S.A.). All other chemicals were commercial products of analytical grade.

cDNA Sequence Identities to the Reference Genes

cDNAs of mouse GAT1 and GAT3 were subcloned into pCMV-SPORT6 and pcDNA-DEST40 vector, respectively. Mutations were corrected using QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, U.S.A.). The sequences of mouse Slc6a cDNAs used in this study were confirmed to be consistent with the respective reference sequences (GAT1/Slc6a1, NM_178703; TAUT/Slc6a6, NM_009320; GAT3/Slc6a11, NM_172890; BGT1/Slc6a12, NM_133661) or to have only silent mutations (GAT2/Slc6a13, NM_144512).

Synthesis of Radiolabeled Hypotaurine

Radiolabeled hypotaurine is not commercially available, so we enzymatically synthesized it by the method of Dominy et al. Recombinant His-SUMO-tagged ADO was produced as follows. pET SUMO/ADO vector was transformed to BL21 competent E. coli (Thermo Fisher Scientific) according to the manufacturer’s guideline. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at the final concentration of 1 mM when the absorbance at 600 nm of the culture solution reached 0.6 in order to enhance expression of the target protein. E. coli was harvested by centrifugation at 3000×g for 10 min and the pellets were solubilized by adding buffer containing 250 mM NaCl, 5 mM imidazole, 0.1% Tween and 20 mM Tris–HCl (adjusted to pH 8.0) and by sonication. The sonicated solution was centrifuged at 30000×g for 30 min and the supernatant was purified on a HisTrap HP column (GE Healthcare Life Sciences, Pittsburgh, PA, U.S.A.). The solution containing recombinant ADO was desalted on a PD-10 desalting column (GE Healthcare Life Sciences) and concentrated in an Amicon Ultra-4 10k (Millipore, Bedford, MA, U.S.A.).

[^14C]Hypotaurine synthesis was initiated by mixing[^14C]-cysteamine solution and the recombinant ADO in 50 mM Tris borate buffer, and the mixed solution was incubated for 30 min at 37°C. The reaction was terminated by addition of sulfosalicylic acid (final concentration: 1.7% (w/v)), and the solution was filtered through a 0.22 µm pore size filter.

The synthesized hypotaurine was purified by chromatography under optimized conditions. Unlabeled cysteamine, taurine, and hypotaurine were separated by liquid chromatography on a COSMOSIL HILIC packed column (4.6 mm×250 mm, Nacalai Tesque, Kyoto, Japan) with mobile phase conditions of acetonitrile:20 mM sodium phosphate (pH 7.0) (70:30), 1.0 mL/min. The analyte was derivatized with o-phthalaldehyde reagent by post-column reaction and detected with a fluorescence detector (FL) at 350 nm excitation and 450 nm emission wavelengths (Fig. 1A). For radio-detection, HPLC coupled with radioactivity detector (Gilson, Middleton, WI, U.S.A.) was used. Purchased[^14C]-cysteamine measured with the LC-radioactivity detector showed the same retention time as unlabeled cysteamine measured with LC-FL detection (Figs. 1A, 1B). The mixture of[^14C]-cysteamine and[^14C]hypotaurine obtained by the incubation of ADO and[^14C]-cysteamine was analyzed with the LC-radioactivity system. A significant peak was seen at the same retention time as unlabeled hypotaurine analyzed by LC-FL (Fig. 1C). Therefore, we connected the LC to a fraction collector and fractionated the mixture of[^14C]-cysteamine and[^14C]hypotaurine.[^14C]Hypotaurine was again subjected to LC before being used for uptake studies. We have confirmed that unreacted[^14C]-cysteamine and other[^14C]-labeled chemicals were not detected (Fig. 1D). The synthesized[^14C]hypotaurine was...
stored at −20°C until use and used it within 3 months after the synthesis.

**Cell Culture** HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 µg/mL streptomycin, 2 mM glutamine at 37°C in a humidified CO2-incubator at the concentration of 5% CO2.

**Establishment of T-REX GAT2-293 Cells** Flp-In T-REX 293 cells (Thermo Fisher Scientific) were cultured in DMEM (Nacalai Tesque) supplemented with designated reagent according to the manufacturer’s protocol. Mouse GAT2 cDNA was subcloned into pcDNA5/FRT/TO vector. GAT2/pcDNA5/FRT/TO and pOG vectors were concomitantly transfected into Flp-In T-REX 293 cells and the culture medium was replaced with fresh medium containing 400 µg/mL hygromycin B, and 15 µg/mL blasticidin for 48 h. Hygromycin-resistant cell colonies were cloned and tetracycline-inducible GAT2 expression was confirmed by measuring [3H]GABA uptake activity (GAT2/mSlc6a13 in Fig. 2). Finally, single-colony-derived cells were established as T-REX GAT2-293 cells.

**Uptake Studies in HEK293 Cells and T-REX GAT2-293 Cells** HEK293 cells were seeded in BioCoat™ Collagen I 6-well plate (Corning Inc., Kennebunk, ME, U.S.A.) at the density of 1.2×10^5 cells/well and cultured for 24 h in a humidified 5% CO2 incubator at 37°C. The cells were transfected with an expression vector (0.75 µg/well) containing designated gene cDNA using Lipofectamine 2000 (1.13 µL/well) (Thermo Fisher Scientific). The culture medium was replaced to the fresh one 6 h after transfection, and the cells were cultured for additional 42 h. T-REX GAT2-293 cells were cultured in the presence of 1 µg/mL tetracycline for 24 h to induce GAT2 expression. The cells were washed 3 times and preincubated with extracellular fluid (ECF) buffer containing 122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM d-glucose and 10 mM 2-[4-(2-hydroxyethyl)piprazinyl]ethanesulfonic acid (HEPES) adjusted at pH 7.4. The uptake reaction was initiated by replacing the medium with fresh medium containing a designated radiolabeled compound. At a designated time, the medium was replaced with fresh medium 3 times to terminate the reaction. The cells were solubilized by adding 1 N NaOH and incubated overnight. The solution was neutralized by adding 1 N HCl, and an aliquot was mixed with scintillation cocktail Clear scint (Nakalai Tesque). The radioactivity was measured with a liquid scintillation counter (LSC-6101, Hitachi-Aloka, Tokyo, Japan). Protein concentration in the remaining solution was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific).

**Cell Proliferation, Antioxidant Capacity and DNA Fragmentation Assay** For cytotoxicity and relative hydroxyl radical antioxidant capacity (HORAC) assays, we dialyzed FBS in a Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific) to remove hypotaurine and other low-molecular physiological metabolites. T-REX GAT2-293 cells were cultured in medium supplemented with the dialyzed FBS in the absence and presence of 1 µg/mL tetracycline for 24 h, and then further cultured in the presence and absence of 0.1 mM hypotaurine for 1 h. Hypotaurine was washed out by replacing the medium with fresh medium, and HORAC was measured with a kit (Oxford Biomedical Research, Rochester Hills, MI, U.S.A.), or the cells were exposed to 0.5 mM H2O2 for 15 min.

After washing out H2O2, the cells were further cultured for 2 h and relative viable cell number was evaluated with the WST-8 assay (Cell Count Reagent SF, Nacalai Tesque).

For DNA fragmentation assay, the cells were treated with 0.1 mM hypotaurine for 1 h as described above and further cultured in the presence of 0.1 mM H2O2 for up to 24 h. DNA fragmentation was analyzed using a DNA fragmentation assay kit (ApopLadder Ex, TaKaRa Bio Inc., Kusatsu, Shiga, Japan).

**Data Analysis and Statistics** Kinetic analysis for estimating Michaelis constant, K_m, was conducted according to Michaelis–Menten equation and the inhibition constant, K_i, was estimated on the assumption that hypotaurine competitively inhibits GAT activity.

\[
V = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

where \(V_{\text{max}}\), \(K_m\), \(K_i\), \(V\), \(S\) and \(I\) represent maximum uptake velocity, Michaelis constant, inhibition constant, uptake velocity, substrate concentration and inhibitor concentration, respectively. Fitting was performed with a nonlinear least-squares regression program using GraphPad Prism4.0 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are presented as mean±standard error of the mean and statistical analysis was performed with Student’s t-test for comparison of two means or one-way ANOVA with Tukey’s post hoc multiple comparison tests in other cases. A p value less than 0.05 was regarded as significant.

**RESULTS**

The results of in-house preparation of [14C]hypotaurine (see Materials and Methods) are shown in Fig. 1. Next, we measured the uptake of [3H]GABA, [14C]hypotaurine and [3H]hypotaurine by cells expressing mouse GAT1, TAUT, GAT3, BGT1 and GAT2. GAT1 mediated significant uptake of [3H]GABA and [14C]hypotaurine, though the uptake of [14C]hypotaurine was 10 times less than that of [3H]GABA (Fig. 2). TAUT mediated significant uptake of [14C]hypotaurine and [3H]TAUT, and [3H]GABA was taken up to a lesser extent. GAT3 mediated significant uptake of [3H]GABA and [14C]hypotaurine, though the uptake of [14C]hypotaurine was 5 times less than that of [3H]GABA (Fig. 2). BGT1 mediated significant uptake of [3H]GABA and [14C]hypotaurine, together with very weak uptake of [14C]-hypotaurine (Fig. 2). GAT2 mediated significant uptake of [3H]GABA, [14C]hypotaurine and [3H]TAUT, with the uptake of the former two compounds being 2 times higher than that of [3H]TAUT (Fig. 2). These results suggest that TAUT and GAT2 are the major hypotaurine transporters.

Measurement of the affinity of hypotaurine for TAUT and GAT2 yielded Michaelis constant (K_m) values of 10.7±1.7 and 10.6±2.1 µM, respectively (Fig. 3). Although GAT3 showed little uptake of hypotaurine, we examined whether hypotaurine inhibited GABA uptake by GAT3. An Eadie–Scatchard plot showed a decrease of apparent K_m value for GABA uptake in the presence of 10 µM hypotaurine. The K_m value for GABA uptake and the K_i value of hypotaurine for GABA uptake were estimated to be 2.24±0.18 and 4.24±0.55 µM, respectively.
To clarify the physiological relevance of GAT2, we used a tetracycline (Tet)-inducible GAT2 stable transfectant (T-REx GAT2-293) cell line established from Flp-In T-REx 293 cells. T-REx GAT2-293 cells were cultured in the absence (mock cells) or presence (GAT2-expressing cells) of tetracycline. The cells were treated with hypotaurine for 1 h and antioxidant capacity was measured as shown in Fig. 5A. The expression of GAT2 significantly increased the antioxidant capacity for hydroxyl radical in the presence of hypotaurine. Hypotaurine-treated cells were exposed to H$_2$O$_2$ for 15 min, and the cell viability was measured after 2 h. Exposure to H$_2$O$_2$ reduced the viable cell count, but tetracycline-induced GAT2 expression significantly ameliorated the cytotoxicity (Fig. 5B). GAT2-expressing cells without hypotaurine pretreatment did not show the increase of viable cell count (data not shown). Further, we measured the antioxidative effect of GAT2-mediated hypotaurine uptake by means of DNA fragmentation assay. Fragmentation of genomic DNA was evident in parent Flp-In T-REx cells exposed to H$_2$O$_2$ for 24 h (Fig. 5C), but the fragmentation was mitigated by GAT2 induction in the presence of hypotaurine (Fig. 5D). These results indicate that
GAT2 expression in the presence of hypotaurine can have antioxidant and cytoprotective effects.

**DISCUSSION**

We have shown that GABA/taurine transporters GAT1, GAT2, GAT3, BGT1 and TAUT can mediate uptake of hypotaurine, and among them, GAT2 and TAUT show the highest uptake activity and affinity. Although GAT2 and TAUT have been identified as GABA and taurine transporters,\(^{20,21}\) this is the first report to show that they also work as hypotaurine transporters. The results are summarized in Table 1. The \(K_m\) values of hypotaurine for TAUT and GAT2 were both approximately 11 \(\mu M\) (Fig. 2, Table 1), and are of the same order as the plasma concentration of hypotaurine, 1–5 \(\mu M\),\(^{9,11}\) implying the physiological relevance of hypotaurine uptake via TAUT and GAT2. The plasma concentration of taurine, 0.4–0.6 mM,\(^{22,23}\) is approximately 100 times higher than the \(K_m\) value of TAUT for taurine, 4.5 \(\mu M\), in mice, so that uptake clearance of other substrates such as hypotaurine from plasma by TAUT would be greatly decreased (by more than 95%) compared to that expected in the absence of taurine. On the other hand, the affinity of taurine for GAT2 is much weaker, and the reported \(K_m\) value of GAT2, 540 \(\mu M\), is similar to the plasma concentration of taurine (Table 1), suggesting that the inhibitory effect of plasma taurine on transport activity of GAT2 for other substrates would be only moderate compared to that in the case of TAUT. Therefore, it is likely that GAT2 plays the predominant role in hypotaurine uptake from the systemic circulation, even though TAUT is expressed at similar levels to GAT2 in various organs.

GAT2 may contribute to physiological hypotaurine transport in several tissues. For example, hypotaurine uptake in mouse brain was well studied by Kontro and Oja in the 1980s.\(^{13,25}\) They reported that the affinity of hypotaurine and GABA for mouse brain slices was 35 and 31 \(\mu M\), respectively, Fig. 4. Inhibitory Effect of Hypotaurine on Mouse GAT3-Mediated [\(^{3}H\)]GABA Uptake

![Fig. 4](image)

**Fig. 4. Inhibitory Effect of Hypotaurine on Mouse GAT3-Mediated \([^{3}H]\)GABA Uptake**

T-REx/GAT2-293 cells were cultured in the presence and absence of tetracycline. Cellular uptake of \([^{3}H]\)GABA at various concentrations was measured for 5 min in the presence (○) and absence (●) of 10 \(\mu M\) hypotaurine. An Eadie–Scatchard plot is shown. Data are presented as the mean±standard error of 3 determinations.

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**Fig. 4. Inhibitory Effect of Hypotaurine on Mouse GAT3-Mediated \([^{3}H]\)GABA Uptake**

T-REx/GAT2-293 cells were cultured in the presence and absence of tetracycline (Tet) to induce GAT2 expression for 24 h, and the cells were exposed to 0.1 mM hypotaurine (HTAU) for 1 h. Hypotaurine was washed out, and the hydroxyl radical antioxidant capacity (HORAC) of the cells was measured by HORAC (A) assay, or the cells were treated with 0.5 mM \(H_2O_2\) for 15 min and then further cultured in fresh medium for 2 h. Viable cell count was determined with WST-8 (B). Data are presented as the mean±standard error of 3 determinations. Statistical significance (\(p<0.05\)) was evaluated by Student’s t-test. DNA fragmentation was measured in Flp-In T-REx293 cells exposed to 0.1 mM \(H_2O_2\) for up to 24 h (C). T-REx/GAT2-293 cells were precultured in the presence and absence of tetracycline and 0.1 mM hypotaurine, and the cells were subsequently cultured in 0.1 mM \(H_2O_2\) for 24 h, then DNA fragmentation was evaluated (D).

**Fig. 5. Cytoprotective Effect of Mouse GAT2 against \(H_2O_2\)-Induced Oxidative Stress**

![Fig. 5](image)

**Table 1. Affinity of Hypotaurine for Mouse Slc6a/GABA Transporters**

<table>
<thead>
<tr>
<th>Affinity ((\mu M))</th>
<th>GABA</th>
<th>Taurine</th>
<th>Hypotaurine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m)</td>
<td>(K_i)</td>
<td>(K_m)</td>
</tr>
<tr>
<td>GAT1/Slc6a1</td>
<td>5.9(^{(35)})</td>
<td>13(^{(36)})</td>
<td>4.5(^{(24)})</td>
</tr>
<tr>
<td>TAUT/Slc6a6</td>
<td>1500(^{(21)})</td>
<td></td>
<td>1400(^{(27)})</td>
</tr>
<tr>
<td>GAT3/Slc6a11</td>
<td>0.8–2.2(^{(12)})</td>
<td>12(^{(38)})</td>
<td>4.9(^{(13)})</td>
</tr>
<tr>
<td>BGT1/Slc6a12</td>
<td>79(^{(7)})</td>
<td>97(^{(8)})</td>
<td>540(^{(7)})</td>
</tr>
<tr>
<td>GAT2/Slc6a13</td>
<td>18(^{(7)})</td>
<td>9.2(^{(38)})</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Determined in this study. \(^{b}\) Estimated from Liu et al., 1992.\(^{26}\)
being greater than that of taurine, 106 μM.\textsuperscript{10} These values are consistent with the putative function of GAT2. Furthermore, GAT2 is expressed in a subpopulation of brain blood vessels, and is proposed to be involved at least in brain-to-blood efflux of taurine.\textsuperscript{17,20,26} GAT2 is expressed on the sinusoidal membrane of perportal hepatocytes.\textsuperscript{20,27} Knockout of the GAT2 gene, \textit{Slc6a13}, in mice reduced the taurine concentration in the liver by approximately 50%.\textsuperscript{20} Thus, GAT2 is functional in the liver, at least in terms of taurine uptake. Our findings suggest that it also contributes to hypotaurine uptake in mouse liver. Although GAT2 is functionally less important for taurine uptake in the kidney, it is expressed in the basolateral membrane of proximal tubules,\textsuperscript{20} and also in the retinal pigment epithelium.\textsuperscript{28,29} Thus, hypotaurine may be taken up by GAT2 in the kidney and the retina. However, it remains unclear whether GAT2 takes up taurine itself, or takes up hypotaurine, which is subsequently converted to taurine. Whatever its role in taurine uptake, it is still possible that GAT2 has an antioxidative function via hypotaurine uptake. Indeed, GAT2 expression enhanced cytoprotection against oxidative stress via hypotaurine uptake (Figs. 2, 5).

Hypotaurine is abundant in developmental tissues such as spermatozoa and in fetal plasma.\textsuperscript{7,12} These tissues are exposed to high levels of oxidative stress and they require antioxidants to protect themselves. Supplementation of hypotaurine improves motility and/or viability of post-thaw semen in a species-specific manner.\textsuperscript{30–32} Functional expression of GABA transporter in human semen has been reported, and the \textit{K}_m value of GABA uptake was 14 μM,\textsuperscript{33} which is close to that of GAT2. It is possible that GABA transporter(s) contribute to hypotaurine uptake in spermatozoa. In the case of pregnant mice, administration of hypotaurine to maternal mice resulted in an increase of hypotaurine concentration in maternal plasma from 5 to 15 μM and in fetal plasma from 125 to 192 μM, indicating that hypotaurine can be transferred from mother to fetus, at least in mice, and the transport process \textit{via} placenta is saturable.\textsuperscript{9} Placental trophoblasts showed hypotaurine uptake activity and the intracellular hypotaurine level was dependent on hypotaurine concentration in the extracellular fluid.\textsuperscript{12} Therefore, it is also possible that uptake transporters such as GAT2 and TAUT contribute to fetal transfer of hypotaurine across placenta.

GAT1, GAT3 and BGT1 also showed hypotaurine uptake ability, but their activities were only slight (Fig. 2). The low uptake activities of GAT1 and BGT1 can be understood in terms of their weak affinity for hypotaurine. As summarized in Table 1, the reported IC\textsubscript{50} values of hypotaurine for GAT1 and BGT1 were much higher than the affinities for GABA and TAUT, implying that the contributions of GAT1 and BGT1 to hypotaurine transfer are minimal, at least in tissues that express GAT2 and/or TAUT. Interestingly, the affinity of GAT3 for hypotaurine based on the \textit{K}_m value was poten even compared to GAT2 and TAUT (Fig. 4), though GAT3 is much more selective for GABA (Fig. 2, Table 1). Compared to GAT2, the tissue distribution of GAT3 is limited, but GAT3 is highly expressed in the brain, particularly in the olfactory bulb, thalamus, hypothalamus, pons and medulla.\textsuperscript{17,26,34} It is possible that GAT3 has a role in hypotaurine transport in the central nervous system.

In conclusion, we have shown that GABA transporters GAT1, GAT2, GAT3, BGT1 and TAUT take up hypotaurine as a substrate, though the uptake activity and affinity of GAT2 and TAUT are much higher than those of GAT1, GAT3 and BGT1. Expression of GAT2 results in an antioxidative effect in the presence of hypotaurine. These findings suggest that hypotaurine uptake mediated by GAT2 and TAUT is physiologically significant.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

15) Pasantes-Morales H, Morán J, Fellman JH. Hypotaurine uptake by...
25) Oja SS, Kontro P. Hypotaurine uptake by brain slices from adult
Ikegaki N, Saito N, Hashima M, Tanaka C. Production of spe-
22) Rozen R, Scriver CR. Renal transport of taurine adapts to perturbed
24) Liu QR, López-Corcuera B, Nelson H, Mandiyan S, Nelson N. Mo-
23) Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A,
17) Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. Mo-
18) Kragler A, Höfner G, Wanner KT. Novel parent structures for in-
16) Holopainen I, Kontro P, Frey HJ, Oja SS. Taurine, hypotaurine, and
17) Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. Mo-
20) Zhou Y, Holmseth S, Guo C, Hassel B, Höfner G, Huitfeldt HS,
Wanner KT, Danbolt NC. Deletion of the γ-aminobutyric acid trans-
21) 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
20) Zhou Y, Holmseth S, Guo C, Hassel B, Höfner G, Huitfeldt HS,
Wanner KT, Danbolt NC. Deletion of the γ-aminobutyric acid trans-
porter 2 (GAT2 and SLC6A13) gene in mice leads to changes in
(2012).
19) Dominy JE Jr, Simmons CR, Hirschberger LL, Hwang J, Co-
loso RM, Stipanuk MH. Discovery and characterization of a second
mammalian thiol dioxygenase, cysteamine dioxygenase. J. Biol.
18) Kragler A, Höfner G, Wanner KT. Novel parent structures for in-
hibitors of the murine GABA transporters mGAT3 and mGAT4.
17) Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. Mo-
22) Rozen R, Scriver CR. Renal transport of taurine adapts to perturbed
(1982).
23) Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A,
Seeliger MW, Warskulat U, Häussinger D. Disruption of the taurine
transporter gene (tauT) leads to retinal degeneration in mice. FASEB
Cloning and expression of a cDNA encoding the transporter of taur-
25) Oja SS, Kontro P. Hypotaurine uptake by brain slices from adult
26) Ikegaki N, Saito N, Hashima M, Tanaka C. Production of spe-
cific antibodies against GABA transporter subtypes (GAT1, GAT2,
GAT3) and their application to immunocytochemistry. Brain Res.
K. γ-Aminobutyric acid transporter 2 mediates the hepatic uptake of
guanidinoacetate, the creatine biosynthetic precursor, in rats. PLOS
28) Honda S, Yamamoto M, Saito N. Immunocytochemical localization
of three subtypes of GABA transporter in rat retina. Brain Res.
29) Johnson J, Chen TK, Rickman DW, Evans C, Brecha NC. Multiple
γ-aminobutyric acid plasma membrane transporters (GAT-1, GAT-2,
30) Bucak MN, Tuncer PB, Sariöztan S, Ulutaş PA, Coyan K, Baspınar
N, Ozkalp B. Effects of hypotaurine, cysteamine and aminoacids
solution on post-thaw microscopic and oxidative stress parameters
31) Cabrita E, Ma S, Diogo P, Martínez-Paramo S, Sarasquete C, Dinis
MT. The influence of certain aminoacids and vitamins on post-thaw
fish sperm motility, viability and DNA fragmentation. Anim. Re-
32) Partyka A, Kodak O, Bajzert J, Kochan J, Nizaiński W. The effect of
γ-carinine, hypotaurine, and taurine supplementation on the quality
33) Aanesen A., Fried G, Andersson E, Gottlieb C. Carrier-mediated
gamma-aminobutyric acid uptake in human spermatozoa indicating
the presence of a high-affinity gamma-aminobutyric acid transport
34) Borden LA, Smith KE, Hartig PR, Branchek TA, Weinshank RL.
Molecular heterogeneity of the gamma-aminobutyric acid (GABA)
transport system. Cloning of two novel high affinity GABA trans-
35) Liu QR, Mandiyan S, Nelson H, Nelson N. A family of genes en-
36) Thomsen C, Sørensen PO, Egebjerg J. 1-(3-[9H-carbazol-9-yl]-
1-propyl)-4-(2-methoxyphenyl)-4-piperidinol, a novel subtype
selective inhibitor of the mouse subtype II GABA-transporter. Br. J.
Expression of a mouse brain cDNA encoding novel gamma-aminobu-