Cloning and Characterization of a Functional Human γ-Aminobutyric Acid (GABA) Transporter, Human GAT-2*

Bolette Christiansen‡, Anne-Kristine Meinild§, Anders A. Jensen∥, and Hans Brøuner-Osborne‡*

From the ‡Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences and §Department of Molecular Biology, Faculty of Sciences, University of Copenhagen, DK-2100 Copenhagen, Denmark

Plasma membrane γ-aminobutyric acid (GABA) transporters act to terminate GABA neurotransmission in the mammalian brain. Intriguingly four distinct GABA transporters have been cloned from rat and mouse, whereas only three functional homologs of these transporters have been cloned from human. The aim of this study therefore was to search for this fourth missing human transporter. Using a bioinformatics approach, we successfully identified and cloned the full-length cDNA of a so far uncharacterized human GABA transporter (GAT). The predicted protein displays high sequence similarity to rat GAT-2 and mouse GAT3, and in accordance with the nomenclature for rat GABA transporters, we therefore refer to the transporter as human GAT-2. We used electrophysiological and cell-based methods to demonstrate that this protein is a functional transporter of GABA. The transport was saturable and dependent on both Na⁺ and Cl⁻. Pharmacologically the transporter is distinct from the other human GABA transporters and similar to rat GAT-2 and mouse GAT3 with high sensitivity toward GABA and β-alanine. Furthermore the GABA transport inhibitor (5)-SNAP-5114 displayed some inhibitory activity at the transporter. Expression analysis by reverse transcription-PCR showed that GAT-2 mRNA is present in human brain, kidney, lung, and testis. The finding of the human GAT-2 demonstrates for the first time that the four plasma membrane GABA transporters identified in several mammalian species are all conserved in human. Furthermore the availability of human GAT-2 enables the use of all human clones of the GABA transporters in drug development programs and functional characterization of novel inhibitors of GABA transport.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (1, 2).

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To whom correspondence should be addressed: Dept. of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark. Tel.: 45-3533-6518; Fax: 45-3533-6040; E-mail: hbo@molpharm.net.

The abbreviations used are: GABA, γ-aminobutyric acid; BGT-1, betaine/GABA transporter-1; l-DABA, l-2,4-diamino-n-butyric acid; DAPA, tL-2,3-diaminoproprionic acid; EF1502, N-(4,4-bis(3-methyl-2-thienyl)-3-butenyl)-3-hydroxy-4-(methylamino)-5,6,7,8-tetrahydridoazoxol[5]isoxazol-3-ol; FMP, FLIPR membrane potential; GAT, GABA transporter; MTC, multiple tissue cDNA; NCB, National Center for Biotechnology Informa-

In the GABAergic synapse, GABA is released from presynaptic nerve terminals, and it exerts its physiological effects through ionotropic GABA_A and GABA_C receptors and metabotropic GABA_B receptors (3, 4). The GABAergic neurotransmission is terminated by rapid uptake of the neurotransmitter from the synaptic cleft into neurons and glial cells by specific high affinity GABA transporters (5–7). To date, four different plasma membrane GABA transporter subtypes have been identified in the central nervous system of several mammalian species (8–17). Unless otherwise specified, we will use the nomenclature introduced by Guastella et al. (8) and Borden et al. (11) for rat and human GABA transporters and refer to the transporters as GABA transporter (GAT)-1, betaine/GABA transporter-1 (BGT-1), GAT-2, and GAT-3. A different nomenclature, originally suggested by Liu et al. (13), names the corresponding homologous transporters in mouse GAT1–GAT4 (without hyphen) respectively.

The GABA transporters belong to the family of Na⁺/Cl⁻–dependent transporters (SLC6 gene family) that also includes transporters for the neurotransmitters dopamine, serotonin, norepinephrine, and glycine (7, 18). The transport process of these transporters is electrogenic because Na⁺ and Cl⁻ is translocated across the membrane together with the respective substrates (7, 19). As an example, the co-transport of one GABA, two Na⁺, and one Cl⁻ has been reported for human (h) GAT-1 (20). The topologies of these membrane-bound proteins are composed of 12 transmembrane domains connected by interchanging extracellular and intracellular loops and intracellular amino and carboxyl termini (8, 9, 21). Recently the crystal structure of a bacterial homolog of the transporters has been resolved, and the structure has brought significant insight into the structure and function of the Na⁺/Cl⁻–dependent transporters (22).

Neurotransmitter transporters are drug targets for several neurological and psychiatric disorders (19, 23). Diseases such as epilepsy, anxiety disorders, schizophrenia, drug addiction, and various pain states are related to the GABA system (24–26), and pharmacological inhibition of GABA transport constitutes an attractive approach to increase overall GABA neurotransmission (27, 28). So far, this concept has been exploited for the treatment of epilepsy where the GAT-1-selective inhibitor tiagabine is administered clinically as adjunctive therapy for partial seizures (29, 30). Furthermore inhibition of GABA transduction; SLC6A13, solute carrier family 6 (neurotransmitter transporter, GABA), member 13; THPO, 4,5,6,7-tetrahydrooxazolo[4,5-c]pyridin-3-ol; m, mouse; h, human; r, rat; MES, 4-morpholineethanesulfonic acid.
transport in general and tiagabine in particular has been suggested for the treatment of clinical conditions such as ischemia, anxiety, sleep disorders in the elderly, and neuropathic pain (31–34).

GAT-1 and GAT-3 are abundantly expressed throughout the rat, mouse, and human central nervous system and absent from the periphery (11–13, 28, 35–39), and thus these two subtypes have attracted considerable attention as potential drug targets (30, 35, 40). Mouse, rat, and human BGT-1 is expressed in both the brain and periphery (10, 14, 16, 17) and believed to be involved in osmoregulation (18) but has recently also been suggested to play a role in the control of epilepsy (41–43). Rat (r) GAT-2 and mouse (m) GAT3 is similarly found in the brain as well as in the periphery where it is abundantly expressed in kidney and liver (11, 13, 36, 39, 44).

In contrast to the four identified plasma membrane GABA transporter subtypes in mouse and rat, only three have been characterized in human. These include hGAT-1, hBGT-1, and hGAT-3, whereas the human ortholog of mGAT3 and rGAT-2 has remained enigmatic and typically is referred to as “not cloned” in the literature (35, 45). However, in 2001, the cDNA supposedly encoding for the hGAT-2 was reported to have been cloned, although functional uptake of [3H]GABA in mammalian cells transiently transfected with this cDNA could not be demonstrated (46). Bioinformatics analysis of this putative hGAT-2 sequence revealed that it was likely to be an incomplete cDNA sequence with several truncations. In the present study, we cloned the full-length hGAT-2 and characterized the pharmacology of the transporter in several functional assays.

**EXPERIMENTAL PROCEDURES**

**Materials**—GlutaMAX-I Dulbecco’s modified Eagle’s medium, dialyzed fetal bovine serum, penicillin, streptomycin, Hanks’ balanced salt solution, and bovine serum albumin were purchased from Invitrogen. All buffer reagents were obtained from Sigma-Aldrich. [2,3-3H]GABA (specific radioactivity, 27.6 Ci/mmol) and d-[2,3-3H]Asp (specific radioactivity, 40.0 Ci/mmol) were purchased from GE Healthcare.

β-Alanine, taurine, 1-2,4-diamino-n-butyr acid (L-DABA), quinidine, NNC-711, and (S)-SNAP-5114 were purchased from Sigma-Aldrich. GABA was obtained from Fluka Chemie AG, Buchs SG, (Dübendorf, Switzerland), betaine was from B.A.S. Synteselaboratorium, nipeptic acid was from Aldrich, and DL-2,3-diaminopropionic acid (DAPA) was from TCI Europe nv (Zwijndrecht, Belgium). The following compounds were synthesized in house: 4,5,6,7-tetrahydroisoxazolo(4,5-c)pyrindin-3-ol (THPO) (47), guvacine (48), and N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzol[d]isoxazol-3-ol (EF1502) (49).

**Cloning of hGAT-2**—The I.M.A.G.E. cDNA clone 4612245 was purchased, and subsequent sequencing revealed the presence of the full-length nucleotide sequence encoding for the open reading frame of hGAT-2. The cDNA of hGAT-2 was amplified by PCR using the forward primer 5′-ggggattgtagctagcgtc-3′ and the reverse primer 5′-ctcgtaggctagctc-3′ and subcloned into the mammalian pcDNA5 vector according to the protocol of the manufacturer (pcDNA5/FRT/V5-His TOPO® TA Expression kit, Invitrogen). The sequence of the cDNA and the absence of mutations were confirmed by automated DNA sequencing.

Bioinformatics Analysis of the Protein Sequence of hGAT-2—An alignment of hGAT-2 with the previously reported sequences of hGAT-2 (46), mGAT3 (13), and rGAT-2 (11) was performed using the ClustalW alignment program available at the home page maintained by The European Bioinformatics Institute. Transmembrane segments in the hGAT-2 protein were identified by the hidden Markov model for prediction of transmembrane helices (50). The algorithm is publicly accessible at the Center for Biological Sequence Analysis, Technical University of Denmark through internet services. Furthermore a hydrophobicity analysis using the TMpred program was performed. This algorithm is based on the statistical analysis of TMbase, a data base of naturally occurring transmembrane proteins (51) and is available at the home page maintained by Swiss EMBnet. The overall sequence identity of the predicted amino acid sequence of hGAT-2 to other related GABA transporters was examined by searching the protein data base at the National Center for Biotechnology Information (NCBI) using the BLASTp algorithm.

Expression Analysis—The expression pattern of hGAT-2 mRNA was examined by reverse transcription-PCR using human multiple tissue cDNA (MTC™) panels according to the protocol of the manufacturer (Clontech). The cDNA was amplified by PCR using the forward primer 5′-attgtagctagctcagcccaacagtaagtg-3′ and the reverse primer 5′-atccaggtaccttacaggccggttgc-3′. The primers are complementary to exons 1 and 4, respectively, and PCR with the two primers resulted in a specific band of 533 nucleotides. PCR was performed using Taq polymerase as described by the manufacturer (Promega, Madison, WI) and a PTC-100 thermal cycler (M) Research, Waltham, MA). The reactions were heated to 95 °C for 2 min and then cycled 35 times at 95 °C for 1 min, 65 °C for 30 s, and 72 °C for 1 min. All reactions were carried out in parallel and were run on a 1% agarose gel containing SYBR Safe™ (Invitrogen). Amplification of a 983-bases pair fragment from human glyceraldehyde-3-phosphate dehydrogenase was used as a control.

Expression of hGAT-2 in Xenopus laevis Oocytes—The hGAT-2 and the mGAT3 cDNAs were subcloned by PCR into an expression vector (pEXP-SML) containing 5′- and 3′-untranslated regions of the Xenopus β-globin gene and a poly(A) signal for optimal expression in oocytes using the GATEWAY® technology (Invitrogen). The cDNA was linearized down-stream of the poly(A) signal and in vitro transcribed with the T7 RNA polymerase using the T7-Message Machine® kit from Ambion (Ambion, Inc., Austin, TX). 50 ng of cRNA was injected into defolliculated stage 5-6 X. laevis oocytes, prepared as described in Meinild et al. (52). The oocytes were incubated in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4) at 19 °C for 3–5 days before experiments were performed.

Electrophysiology—The two-electrode voltage clamp method was used to control the membrane potential and monitor the whole cell current in oocytes expressing hGAT-2. The recordings were performed at room temperature with a Dagan Clam- pator (Dagan Corp., Minneapolis, MN) interfaced to a personal
computer using a DigiData 1320 analog/digital converter and pCLAMP 9 (Axon Instruments at Molecular Devices, Sunnyvale, CA). For continuous current measurements, the membrane potential was held at −50 mV, and the currents were low pass-filtered at 1 Hz and sampled at 10 Hz. To obtain steady-state current/voltage relationships the membrane potential was held at −50 mV and jumped to test potentials ranging from +50 to −150 mV in 20-mV increments for 200 ms. Currents were low pass-filtered at 500 Hz and sampled at 2 kHz. In general, the experimental chamber was continuously perfused by a NaCl solution (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, pH 7.4). In Na⁺ substitution experiments, Na⁺ was equimolarly replaced with choline ions, and in experiments with low concentrations of Cl⁻ (6 mM), NaMES was used instead of NaCl. In experiments with low Cl⁻ concentrations, the reference electrode was connected to the experimental chamber via an agar bridge (3% agar in 3 M KCl).

Cell Culture and Transfections—tsA201 cells (a transformed human embryonic kidney 293 cell line) (53) were cultured in GlutaMAX-I Dulbecco’s modified Eagle’s medium supplemented with 10% diazoyl fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The constructs encoding the hGAT-2 and the human excitatory amino acid transporter hEAAT3 were transiently transfected into cells using PolyFect according to the protocol of the manufacturer (Qiagen, West Sussex, UK), and the functional assays were performed 36–48 h later.

[^H]GABA and d-[^H]Asp Uptake Assays—tsA201 cells transfected with hGAT-2-pcDNA5 or hEAAT3-pcDNA3 were split into poly-d-lysine-coated white 96-well plates (BD Biosciences). The next day, the medium was removed, and cells were washed with 100 µl of assay buffer (Hanks’ balanced salt solution supplemented with 20 mM HEPES, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4). Then 75 µl of assay buffer supplemented with [^H]GABA or d-[^H]Asp and various concentrations of the test compounds was added to each well, and the plate was incubated at 37 °C for 3 min. Then the cells were washed with 3 × 100 µl of ice-cold assay buffer, and 150 µl of Microscint™20 scintillation fluid (PerkinElmer Life Sciences) was added to each well. The plate was shaken for at least 1 h and counted in a Packard TopCount microplate scintillation counter.

In the saturation experiments, a [^H]GABA concentration up to 100 nM was used, and to measure transport at higher concentrations the radioligands were diluted with the corresponding “cold” ligand (GABA). Non-specific transport was determined in the presence of 3 mM GABA. In the competition transport experiments, either 30 nM [^H]GABA (in the experiments with hGAT-2) or 30 nM d-[^H]Asp (in the experiments with hEAAT3) was used as tracer concentration. The [^H]GABA competition curves were constructed based on measurements obtained typically for eight different concentrations of the test compounds. The following maximal concentrations of the test compounds were applied: GABA, 3 mM; DAPA, 1 mM; β-alanine, 3 mM; (S)-SNAP-5114, 500 µM; EF1502, 250 µM; nipoctic acid, 10 mM; L-DABA, 3 mM; quinidine, 1 mM; guvacine, 10 mM; NNC-711, 1.6 mM; THPO, 10 mM; taurine, 10 mM; and betaine, 10 mM.

The FLIPR™ Membrane Potential (FMP) Assay—The test compounds were characterized functionally in the FMP assay (Molecular Devices, Crawley, UK) essentially as described previously (54). Briefly tsA201 cells transfected with hGAT-2-pcDNA5 were split into poly-d-lysine-coated black clear bottom 96-well plates (BD Biosciences). The next day, the culture medium was removed, and the cells were washed with 100 µl of assay buffer (same buffer as used in the [^H]GABA uptake assay). In the substrate experiments, 100 µl of assay buffer supplemented with FMP assay dye was added to each well, and the plate was incubated at 37 °C for 30 min. The plate was assayed at 37 °C in a NOVOSTar™ plate reader (BMG Labtechnologies, Offenburg, Germany) measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 25 µl of substrate solution (the substrate was dissolved in assay buffer). Inhibition experiments were performed similarly except that a mixture of 50 µl of FMP assay dye solution (2× final concentration in assay buffer) and 50 µl of inhibitor solution (2× final concentration in assay buffer) was incubated at 37 °C for 30 min, and the plate was assayed by addition of 25 µl of GABA solution (assay concentration of GABA, 50 µM). The experiments were performed in triplicate at least three times for each test compound. The concentration-response curves for the substrates and the concentration-inhibition curves for the inhibitors were constructed based on the maximal responses obtained for the various concentrations of the respective compounds. For generation of concentration-response curves the following maximal concentrations of the test compounds were applied: GABA, 3 mM; β-alanine, 3 mM; L-DABA, 1 mM; nipoctic acid, 10 mM; guvacine, 3 mM; taurine, 10 mM; betaine, 3 mM; and THPO, 3 mM.

Data Analysis—All data were analyzed using Prism 4.0b (GraphPad Software, San Diego, CA). For experiments with X. laevis oocytes, the transporter-specific substrate-induced current (I_{substrate}) was obtained from the difference between the currents in NaCl ± substrate. For steady-state kinetic analysis, the I_{substrate} was measured at various membrane potentials and external substrate concentrations, and at each voltage the I_{substrate} versus concentration of substrate relations were fitted to the Michaelis-Menten equation \( I = (I_{\text{max}} \times [S]) / ([S] + K_{0.5}) \) where [S] is the substrate concentration, \( I_{\text{max}} \) is the maximal current for saturating [S], and the half-maximal concentration, \( K_{0.5} \), is the substrate concentration giving rise to 50% of \( I_{\text{max}} \). The \( K_{0.5} \) for GABA and β-alanine was obtained at 100 mM external Na⁺, varying the GABA or β-alanine concentrations, and Na⁻ activation was obtained at a saturating GABA concentration (100 µM) and varying external Na⁺ concentrations.

The saturation curves from the [^H]GABA uptake assay were fitted by nonlinear regression using the equation \( Y = B_{\text{max}} \times X / (K_{0.5} + X) \) where X is the concentration of the ligand, Y is the specific binding, \( B_{\text{max}} \) is the maximal binding, and the equilibrium dissociation constant, \( K_{0.5} \), is the concentration of ligand required to reach half-maximal binding. The inhibition curves from the [^H]GABA and d-[^H]Asp uptake assays were fitted by nonlinear regression using the equation \( Y = B_{\text{max}} + (Top - Bottom) / (1 + 10^{((log K_{\text{m}} - X) \times Hill slope)}) \), where X is the...
logarithm of the concentration, $Y$ is the response, Top is the $Y$ value at the top plateau, Bottom is the $Y$ value at the bottom plateau, $\log K_m$ is the $X$ value when the response is halfway between Bottom and Top, and the Hill slope describes the steepness of the curve.

The concentration-response curves from the FMP assay were fitted using the same equation as described above for the inhibition curves in the uptake assay. The $K_i$ value of THPO was determined based on the following equation: 

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

where $IC_{50}$ is the concentration of compound that produces 50% of the maximum inhibitory response, $[S]$ is the concentration of substrate (GABA) used for the inhibition experiment, and $K_m$ is the concentration of substrate required to reach half-maximal activation. Determination of $K_m$ for GABA was always performed on the same day as the inhibition experiment.

RESULTS

Cloning of the hGAT-2—Initially we performed a multiple alignment of the amino acid sequence of the previously cloned non-functional hGAT-2 (GenBankTM accession number U76343 (46)) with the mouse and rat orthologs. This comparison revealed that the previously reported hGAT-2 cDNA clone contained four truncations and a fairly different carboxyl-terminal sequence compared with the mouse and rat sequences. Due to these differences we next performed a tBLASTn search of the human genome and expressed sequence tag data bases at the NCBI website using the mGAT3 amino acid sequence as query. Results from both of these data bases suggested that the truncations and deviating carboxyl-terminal sequence compared with the mouse and rat sequences. Due to these differences we next performed a tBLASTn search of the human genome and expressed sequence tag data bases at the NCBI website using the mGAT3 amino acid sequence as query. Results from both of these data bases suggested that the truncations and deviating carboxyl-terminal sequence of the previously reported hGAT-2 cDNA clone was erroneous and that a full-length human ortholog does exist. Further searches of GenBank at NCBI revealed a full-length cDNA clone identified by the Mammalian Gene Collection Program Team
(accession number BC022392 (55)) that could be obtained from the I.M.A.G.E. Consortium (56). We then used PCR to amplify a nucleotide sequence from the I.M.A.G.E. clone that is identical to GenBank accession number BC022392. The cDNA has a predicted amino acid sequence containing an open reading frame of 602 amino acids, which is in good agreement with the lengths of the rat and mouse orthologs (11, 13). Alignment analysis demonstrates that the sequence is different from the truncated hGAT-2 of 569 amino acids previously reported by Gong et al. (46) and that the amino acid sequence of the full-length hGAT-2, reported here, displays high similarity to mGAT3 and rGAT-2 including the carboxyl-terminal sequence (Fig. 1).

Analysis of Sequence—A search in the gene data base maintained by NCBI demonstrated that the gene for hGAT-2 (SLC6A13) is localized to human chromosome 12p13.3. The presence of 14 exons in the gene was found by comparison between the amino acid sequence of hGAT-2 and the genomic DNA (Fig. 2A). These findings are similar to what has recently been reported in a description of the complete repertoire of the SLC6 family (57).

The topology of the predicted protein was analyzed using two different analysis tools for prediction of transmembrane helices. Analysis by the hidden Markov model predicted the presence of 12 transmembrane α-helices (Fig. 2B). Hydrophobicity analysis using the TMpred program identified two possible models for transmembrane topology with 11 and 12 transmembrane α-helices, respectively, depending on the predicted orientation of the membrane spanning segments (Fig. 2C). A similar result was obtained when the TMpred program was run using the amino acid sequences of both mGAT3 and rGAT-2 (data not shown). Collectively the two algorithms demonstrate that hGAT-2 contains 12 putative transmembrane α-helices, which is expected for a member of the Na\(^+\)/Cl\(^-\)-dependent neurotransmitter transporter superfamily (7).

The similarity of the predicted amino acid sequence of hGAT-2 to other GABA transporters was examined. Alignment analysis demonstrated that GAT-2 displays 51–74% overall amino acid identity to the other human GABA transporters and 91% identity to the mouse and rat orthologs mGAT3 and rGAT-2 (Fig. 2D).

Expression Pattern of hGAT-2 mRNA in Human Tissues—To examine the expression pattern of the transporter in human tissues we performed reverse transcription-PCR of commercially available human tissue panels. These MTC panels consist of first strand cDNA generated using poly(A)\(^+\) RNA derived from normal adult human tissues. The cDNA in the applied MTC panels has been normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The reverse transcription-PCR experiment revealed a high mRNA level of hGAT-2 in kidney and low levels in brain and lung. Furthermore we were able to detect a very low level of mRNA of the transporter in testis, whereas it was absent from the other tested peripheral tissues (Fig. 3).

Pharmacological Characterization of hGAT-2 Expressed in X. laevis Oocytes—To determine whether the cloned hGAT-2 cDNA encodes for a functional GABA transporter, we expressed the hGAT-2 construct in X. laevis oocytes. For the characterization of hGAT-2 in this and subsequent assays, we used a number of different compounds (depicted in Fig. 4). Addition of GABA (500 \(\mu M\)) induced an inwardly directed current in voltage-clamped oocytes injected with hGAT-2 cRNA. \(\beta\)-Alanine (500 \(\mu M\)) similarly induced an inward current, whereas betaine (500 \(\mu M\)) did not. The magnitudes of the
GABA- and β-alanine-induced currents were not the same when measured in the same oocyte: $I_{\beta\text{- Ala}} > I_{\text{GABA}}$ (Fig. 5A). In the presence of either GABA or β-alanine there was an almost linear relationship between current and voltage, and the substrate-induced current did not saturate with hyperpolarizing membrane potentials (Fig. 5B).

The kinetics of substrate transport through hGAT-2 was determined by measuring the steady-state substrate-induced currents at different substrate concentrations and constant Na$^+$ and Cl$^-$ concentrations. The substrate-induced currents reached saturation levels with increasing concentrations of substrate, and the currents could be fitted to the Michaelis-Menten equation. The $K_{0.5}$ was $3.7 \pm 0.7 \mu M$ for GABA ($n = 4$) and $20.2 \pm 0.3 \mu M$ ($n = 5$) for β-alanine at a membrane potential of $-90$ mV (Fig. 5, C and D). The $K_{0.5}$ values were not significantly different at membrane potentials ranging from $-150$ to $-30$ mV (Fig. 5E). This $K_{0.5}$ for GABA was similar to the $K_{0.5}$ for GABA in oocytes injected with the mouse ortholog transporter mGAT3 (data not shown and Ref. 58).

It was also investigated whether the GABA-induced currents in hGAT-2-expressing oocytes could be inhibited by a GABA transporter inhibitor. We used oocytes clamped at $-50$ mV, and the results show that inward current induced by 50 μM GABA was partly inhibited by addition of 100 μM (S)-SNAP-5114 to the superfusing solution, whereas addition of 500 μM (S)-SNAP-5114 blocked the GABA-induced current to 8.5 ± 9.0% of the initial inward current ($n = 4$) (Fig. 5F).

In the studies of hGAT-2 expressed in oocytes the substrate transport through the transporter was found to be highly dependent on the external ions. Replacement of 100 mM Cl$^-$ with NaMES in the perfusing solution caused a reduction in the GABA transport, whereas removal of Na$^+$ abolished the GABA-induced current as demonstrated by the replacement of Na$^+$ with choline ions (Fig. 5G). When measured in the same oocyte, a reduction in Cl$^-$ from 106 to 6 mM resulted in a reduction in $I_{\text{GABA}}$ from 100 to 54.4 ± 3.9% ($n = 6$). When Na$^+$ was removed from the external solution, $I_{\text{GABA}}$ decreased to 0.4 ± 4.5% ($n = 5$).

To further investigate the Na$^+$ dependence of the substrate transport we measured the GABA-induced current at different Na$^+$ concentrations and a constant GABA concentration of 100 μM. The GABA-induced current plotted as a function of the external Na$^+$ concentration was fitted to the Michaelis-Menten equation to obtain values for the half-maximal Na$^+$ concentration. For Na$^+$ the $K_{0.5}$ was highly voltage-dependent with a value of 25.6 ± 4.1 mM at $-150$ mV and 93.3 ± 11.1 mM at $-90$ mV. It was not possible to fit the data at membrane holding potentials more positive than $-90$ mV (data...
not shown). In mGAT3 the $K_{0.5}$ was also highly voltage-dependent (data not shown) but 3–4-fold lower than for hGAT-2 (data not shown and Ref. 58).

**Pharmacological Characterization of hGAT-2 in the [3H]GABA Uptake Assay**—Next we characterized the pharmacology of hGAT-2 transiently expressed in tsA201 cells in a [3H]GABA uptake assay. Initial experiments demonstrated a time-dependent uptake of GABA in cells transiently expressing hGAT-2. The uptake was linear up to $\sim$10 min, and thus it was decided to use an uptake period of 3 min for pharmacological analysis as this gave rise to an uptake of less than 10% of the total amount of [3H]GABA in the assay buffer (data not shown).

In the saturation experiments with hGAT-2-expressing tsA201 cells, a dose-dependent increase in [3H]GABA uptake reaching saturation levels at higher concentrations was observed. The saturable transport of GABA was characterized by a $K_m$ value of $8.24 \pm 0.38 \mu M$ ($n = 4$) (Fig. 6A).

The pharmacological properties of 13 standard GABA transporter ligands (Fig. 4) were characterized at the hGAT-2 in the [3H]GABA uptake assay using a tracer concentration of 30 nM [3H]GABA. In this assay, all test compounds displayed dose-dependent inhibition curves with the exception of betaine, which only displayed minor inhibitory activity at concentrations up to 10 mM. GABA, DAPA, and $\beta$-alanine displayed the most potent competition of GABA transport at hGAT-2 ($IC_{50}$ values in the range of 10–100 $\mu M$). The compounds (S)-SNAP-5114, EF1502, nipeptotic acid, DABA, and quinidine were moderate in the competition of [3H]GABA transport ($IC_{50}$ values of 100–800 $\mu M$), whereas guvacine, NNC-711, THPO, taurine, and betaine displayed only low or very low competition of the transporter activity ($IC_{50} \geq 1000 \mu M$) (Table 1 and Fig. 6B). As a control for non-specific inhibition of [3H]GABA uptake, the test compounds were
also characterized at hEAAT3-expressing tsA201 cells. None of the compounds inhibited the uptake of D-[3H]Asp at hEAAT3 in the concentrations used for hGAT-2 (data not shown).

Pharmacological Characterization of hGAT-2 in the FMP Assay—We characterized the functional properties of the 13 test compounds in the fluorescence-based high throughput membrane potential assay. The basis of this assay is the distribution of an assay dye across the cell membrane that is dependent on the membrane potential of the cells. One particular advantage of using this test system is the ability to distinguish between substrates and inhibitors of electrogenic transporters (54).

In the FMP assay exposure of hGAT-2-transfected tsA201 cells to known GABA transporter substrates gave rise to solid concentration-dependent increases in fluorescence intensity (Fig. 7). The increase in the fluorescence intensity in this assay reflects cell depolarization, which again is a reflection of the co-transport of Na\(^+\) and Cl\(^-\) ions through the transporter elicited by the transport of the substrate. GABA, β-alanine, L-DABA, nipeotic acid, guvacine, taurine, and betaine were identified as substrates for the hGAT-2, whereas THPO was found to be a weak inhibitor (Table 2). The remaining test compounds gave rise to nonspecific responses at the hGAT-2 in the FMP assay because they exhibited similar responses in cells transfected with the glutamate transporter hEAAT3 (data not shown). Hence these compounds were not tested further in the FMP assay. The Hill slopes for betaine and THPO could not be compared. ND, not determined.

![Graph](image)

FIGURE 6. Pharmacological characterization of hGAT-2 in the [3H]GABA uptake assay using tsA201 cells transiently transfected with hGAT-2. A, saturation curve for GABA transport where specific uptake of increasing concentrations of GABA was measured. The maximum concentration of [3H]GABA used was 100 nM, and the uptake assay was performed as described under “Experimental Procedures.” Data are given as pmol/well and are means ± S.D. of triplicate determinations of a single representative experiment. The \(K_v\) for GABA was determined to be 8.24 ± 0.38 \(\mu M\) (n = 4). B, representative concentration-inhibition curves of GABA (■), DAPA (○), (S)-SNAP-5114 (▲), guvacine (▲), THPO (▲), and taurine (▲). Competition for transport of 30 nM [3H]GABA by the indicated standard ligands was performed as described under “Experimental Procedures.” Results are given as CPM and are means ± S.D. of triplicate determinations of single representative experiments. At least two additional experiments performed on different days gave similar results.

![Graph](image)

FIGURE 7. Pharmacological characterization of hGAT-2 in the FMP assay using tsA201 cells transiently transfected with hGAT-2. Shown are representative concentration-response curves of GABA (■), β-alanine (○), L-DABA (▲), nipeotic acid (▲), guvacine (▲), taurine (▲), and betaine (▲). The FMP assay was performed as described under “Experimental Procedures.” The responses are given as Δ fluorescence units (ΔFU) and are means of triplicate determinations of single representative experiments. Error bars have been omitted for clarity. At least two additional experiments performed on different days gave similar results.

| Compound | hGAT-2 IC\(_{50}\) (μM) ± S.E. | mGAT3 IC\(_{50}\) (μM) | rGAT-2 IC\(_{50}\) (μM) |
|----------|-------------------------------|---------------------|---------------------|
| GABA     | 10.9 (4.99 ± 0.07)            | 9.2 (3.94 ± 0.07)   | 17 (5.7 ± 0.02)     |
| DAPA     | 28.2 (14.58 ± 0.13)           | 20 (10 ± 0.02)     | 30 (15 ± 0.11)     |
| β-Alanine| 41.9 (44.11 ± 0.12)           | >10 (5 ± 0.01)     | 66 (19 ± 0.06)     |
| EF1502   | 286 (15.55 ± 0.05)            | 20 (10 ± 0.01)     | 21 (10 ± 0.01)     |
| Nipeotic acid | 520 (26 ± 0.05)       | >120 (60 ± 0.01)   | 110 (50 ± 0.01)   |
| 1-DABA   | 633 (32 ± 0.05)               | >500 (250 ± 0.01)  | 300 (150 ± 0.01)  |
| Quinidine| 716 (3.15 ± 0.05)             | >100 (50 ± 0.01)   | ND                 |
| Guvacine | 1000 (3.01 ± 0.08)            | 500 (250 ± 0.01)   | 228 (114 ± 0.01)  |
| NNC-711  | 1210 (1.92 ± 0.01)            | 250 (125 ± 0.01)   | 729 (365 ± 0.01)  |
| THPO     | 1450 (2.84 ± 0.01)            | 800 (400 ± 0.01)   | >100 (50 ± 0.01)  |
| Taurine  | >3000                        | 5400 (2700 ± 0.01)| 1270 (635 ± 0.01) |
| Betaine  | >3000                        | >5000 (2500 ± 0.01)| >5000 (2500 ± 0.01)|

* Thomsen et al. (66).
* Kragler et al. (59).
* Borden et al. (63).
* Borden et al. (15).
* Liu et al. (13).
* The concentration-inhibition curves for compounds that displayed <90% inhibition of hGAT-2 at the maximal tested concentration were fitted to the value of 100% inhibition (1 mM GABA). These compounds exhibited the following maximal inhibition: (S)-SNAP-5114 (500 μM), 80.3 ± 1.3%; EF1502 (250 μM), 47.4 ± 2.1%; L-DABA (3 μM), 77.9 ± 1.9%; quinidine (1 μM), 61.6 ± 4.2%; NNC-711 (1.6 μM), 58.9 ± 3.9%; THPO (10 μM), 87.6 ± 1.0%; taurine (10 μM), 74.1 ± 0.2% betaine (10 μM), 21.3 ± 1.6%.
* Clausen et al. (49).
* Borden et al. (61).
* Bolvig et al. (62).
* Borden et al. (11).
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estimated; however, for the rest of the tested substrates the average Hill slopes were between 1.2 and 1.7.

DISCUSSION

Since the cloning of the rat, mouse, and human GABA transporters in the early 1990s, the possible existence of hGAT-2 remained enigmatic until a non-functional clone was reported in 2001 (46). Our analysis of this sequence revealed that it contains several truncations and a deviating carboxyl terminus compared with the rat and mouse orthologs; this was puzzling considering the close sequence relationship shared by the orthologs of the other three GABA transporter subtypes. This observation led us to use bioinformatics tools to identify the full-length open reading frame of hGAT-2 containing a carboxyl terminus similar to the rat and mouse orthologs. Here we thus report the cDNA cloning of the human GABA transporter hGAT-2, which is functional and displays pharmacological characteristics very similar to its rat and mouse orthologs rGAT-2 and mGAT3. The protein is closely related to other cloned human plasma membrane GABA transporters and displays high amino acid sequence similarity to mGAT3 and rGAT-2. Examination of mRNA levels of hGAT-2 by reverse transcription-PCR in tissues from adult individuals revealed expression in kidney, brain, lung, and testis. This corresponds to previous reports using either Northern blots (46) or analysis of expressed sequence tags (57), which also demonstrate expression of hGAT-2 in kidney and brain. Furthermore expression in liver (46) and in eye and the gastrointestinal system has been reported (57). The rGAT-2 has been detected in brain and retina, and in peripheral tissues it has been found in kidney, liver, heart, spleen, pancreas, and adrenal gland (11, 36, 39). The expression of mGAT3 has been found to be more restricted with low abundance in adult mouse brain and higher levels of expression in kidney and liver. Interestingly expression at a high level has been found in neonatal mouse brain (13), and the expression of mGAT3 in various embryonic peripheral tissues has also been reported (44). Based on the expression pattern, several suggestions regarding a physiologically relevant function of GAT-2 have been made. These include maturation of the central nervous system (13, 44), regulation of GABA levels in the cerebrospinal fluid (36, 37, 39), and a role for GAT-2 in tight junctions in the kidney (44).

Using the *Xenopus* oocyte expression system we demonstrated that hGAT-2 is a functional transporter. Putative substrates (GABA and β-alanine) induced inwardly directed currents under voltage-clamped conditions, reflecting the co-transport of ions and substrates. Betaine, however, is not a substrate for hGAT-2, as would be expected for this transporter (in contrast to the BGT-1). Not surprisingly, the kinetics of the hGAT-2 are comparable to those of the ortholog mGAT3 (58), whereas they differ from those of hGAT-1 (20). Examination of ion dependence revealed that the transport of substrate is absolutely dependent on Na⁺, whereas transport is not completely eliminated upon removal of Cl⁻. This is in agreement with observations made for both mGAT3 and rGAT-2 and also hGAT-1 where it has been reported that the transporters are strictly dependent on the presence of Na⁺, whereas the absence of Cl⁻ only reduces GABA transport by ~50% (11, 20, 58).

We examined the pharmacological characteristics of hGAT-2 transiently expressed in tsA201 cells using a number of both synthesized and commercially available compounds (Fig. 4). These compounds have been tested previously as substrates or inhibitors of GABA transport and found to possess different selectivity profiles for neuronal and glial transport and for the four cloned GABA transporters. The compounds include the classical GABA transporter substrates L-DABA and β-alanine, which originally were thought to be markers for neuronal and glial transport, respectively, as well as nipeptic acid and guvacine, which were reported to be substrates at both neuronal and glial GABA transporters (for a review, see Ref. 27). Also included in this study is DAPA, which has been reported to be selective for the mouse orthologs of GAT-2 and GAT-3 (13, 59); quinidine and betaine, which display selectivity especially the mouse ortholog of BGT-1 (10, 13, 14, 28); (S)-SNAP-5114, which is a relatively selective inhibitor of hGAT-3 (12, 60); EF1502, which preferentially inhibits the mouse orthologs of GAT-1 and BGT-1 (41); NNC-711, and which selectively inhibits rGAT-1 and hGAT-1 (61); THPO, which is a weak inhibitor of all the cloned mouse GABA transporters (62); and taurine, which is a selective substrate of the closely related taurine transporter in rat, TAUT (63, 64).

Applying the [³H]GABA uptake assay and the FMP assay we demonstrated that hGAT-2, similarly to hGAT-1 and hGAT-3, displays high affinity transport of GABA. Furthermore we found the approximately same orders of potencies for the tested substrates in the two assays: GABA ≥ β-alanine > L-DABA = nipeptic acid ≥ guvacine > taurine = betaine. In agreement with the oocyte data (Fig. 5A) and previous reports (see Refs. 27, 62, and 65 and references therein) the results demonstrate that the applied FMP assay can be used to distinguish between substrates and non-substrates at GABA transporters. However, due to nonspecific effects the pharmacological properties of the compounds DAPA, (S)-SNAP-5114, quinidine, NNC-711, and EF1502 unfortunately could not be studied in the FMP assay. Considering the bulky substituents of the four latter compounds, it is reasonable to assume that they are inhibitors of hGAT-2 just as they have been demonstrated to be at other

| Substrates | $K_m$ (pM) ± S.E. | $R_{max}$ ± S.E.* | $n$ |
|------------|------------------|------------------|-----|
| GABA       | 18.3 (4.75 ± 0.03) | 100              | 12  |
| β-Alanine  | 39.3 (4.43 ± 0.10) | 142.4 ± 2.6      | 3   |
| L-DABA     | >200             | 120.9 ± 9.4      | 3   |
| Nipectic acid | 288 (3.54 ± 0.04) | 96.5 ± 24        | 3   |
| Guvacine   | >300             | 139.2 ± 5.3      | 3   |
| Taurine    | >1000            | 150.2 ± 4.6      | 3   |
| Betaine    | >1000            | 205.0 ± 6.6      | 3   |

| Non-substrate inhibitor | $K_J$ (pM) ± S.E. | Inhibition ± S.E.* | $n$ |
|-------------------------|------------------|-------------------|-----|
| THPO                    | >200             | 49.8 ± 3.5        | 3   |

* The maximum responses are normalized to the maximal response of GABA (set at 100%) measured at 1 mM.

* The concentration-response curve did not reach a plateau at the maximal measured concentration of the compound. The $K_m$ or $K_i$ value is therefore estimated based on the maximal measured response/inhibition of the compound.

* Percent inhibition of the response elicited by 50 μM GABA by THPO at a concentration of 3 mM.

![Table 2](image-url)
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GABA transporter subtypes as described above. Intriguingly several of the substrates (e.g. β-alanine, guvacine, and i-DABA) displayed maximal responses greater than the maximal response of GABA (Table 2 and Fig. 7). This observation cannot be interpreted as an increased substrate transport rate for these compounds compared with GABA because other events could change the ion flux ratios during the interaction of the compounds with the transporter (54). However, the increased response level is an indication of increased currents, which is in agreement with the observed current trace for β-alanine in the Xenopus oocyte expression system (Fig. 5A).

Comparing the pharmacological characteristics found in this study (Tables 1 and 2) with reports for hGAT-1, hBGT-1, and hGAT-3, most of the results obtained in this study are in good agreement with previously reported properties of the compounds. Betaine, quinidine, taurine, NNC-711, and THPO all display low activities at hGAT-2. Nippecotic acid and guvacine display weak to moderate activities as substrates in the FMP assay and as competitors of [3H]GABA transport at hGAT-2. In comparison these two compounds have been reported to display strong competition of [3H]GABA transport at hGAT-1 and moderate and weak competition at hGAT-3 and hBGT-1, respectively (12). The hGAT-2 and hGAT-3 both display high affinity transport of β-alanine, whereas (S)-SNAP-5114 is a selective inhibitor of hGAT-3-mediated transport (12) and displays a substantially lower inhibitory activity at hGAT-2. Our study therefore demonstrates that hGAT-2, in agreement with the overall amino acid identity (Fig. 2D), is different from the other cloned GABA transporters and that the pharmacology of the transporter is more similar to hGAT-3 and hBGT-1 than to hGAT-1.

As can be seen from Table 1, the overall rank order of IC₅₀ values for the 13 standard compounds at hGAT-2 and its rat and mouse orthologs rGAT-2 and mGAT3 is similar. This is hardly surprising considering the high amino acid sequence similarity between the three transporters. (S)-SNAP-5114 appears to display a lower inhibitory activity at hGAT-2 compared with mGAT3 and rGAT-2. However, it should be noted that data in Table 1 are derived from several different studies, and caution should therefore be taken when drawing comparisons because differences between transporters from different species also could arise from different assay conditions, assay systems, or expression levels. This is also indicated by the variation of the inhibition constants measured for some of the compounds at either rGAT-2 or mGAT3 (Table 1).

The therapeutic potential of targeting the hGAT-2 remains to be clarified. It has been suggested previously to be unlikely that GAT-2 is involved in regulation of GABA inside and outside intracerebral synapses (35). However, it may be noted that BGT-1, which similarly to GAT-2 is expressed in non-neural tissues, only recently has been suggested to have a functional role in the central nervous system (41). This finding was based on the discovery of the novel GABA transport inhibitor EF1502, which acts on both GAT-1 and BGT-1 (49). So far, (S)-SNAP-5114 is the only available pharmacological tool for determination of the functional role of especially GAT-3 but potentially also GAT-2 in the central nervous system (28). However, the recently obtained knowledge about the determinants of substrate binding to a homolog of the GABA transporters (22) is likely to enable a more rational approach to the design of new selective compounds for each of the GABA transporters. Such compounds will hopefully reveal more about the function of GAT-2 both in the central nervous system and in the periphery. Furthermore the identification of this human ortholog of rGAT-2 and mGAT3 enables the use of the human GABA transporters in future studies of GABA transport inhibitors. Although GAT-2 is similar among the species mentioned in this study, there appears to be some variations in transporter pharmacology, and thus the availability of hGAT-2 is highly relevant for future drug development programs.

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