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Cloning and Expression of a Rat Brain GABA Transporter

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A complementary DNA clone (designated GAT-1) encoding a transporter for the neurotransmitter γ-aminobutyric acid (GABA) has been isolated from rat brain, and its functional properties have been examined in *Xenopus* oocytes. Oocytes injected with GAT-1 synthetic messenger RNA accumulated [^{3}H]GABA to levels above control values. The transporter encoded by GAT-1 has a high affinity for GABA, is sodium- and chloride-dependent, and is pharmacologically similar to neuronal GABA transporters. The GAT-1 protein shares antigenic determinants with a native rat brain GABA transporter. The nucleotide sequence of GAT-1 predicts a protein of 599 amino acids with a molecular weight of 67 kilodaltons. Hydropathy analysis of the deduced protein suggests multiple transmembrane regions, a feature shared by several cloned transporters; however, database searches indicate that GAT-1 is not homologous to any previously identified proteins. Therefore, GAT-1 appears to be a member of a previously uncharacterized family of transport molecules.

THE PLASMA MEMBRANES OF NEURONS and glia, and the synaptic vesicle membranes of neurons, have ion-dependent transporters capable of bidirectional neurotransmitter transport. The plasma membrane transporters probably terminate synaptic activity, although other functions have been proposed. Neurotransmitter transporters have been extensively studied pharmacologically, but the molecular features of these proteins are largely unknown.

The amino acid GABA is the predominant inhibitory neurotransmitter in the mammalian brain. Plasma membrane transporters for GABA are widely distributed in the central and peripheral nervous systems (*1, 2*). Pharmacological and kinetic studies suggest the presence of a variety of GABA transporter subtypes (*1–3*). Thus, GABA transporters may make up a family of proteins possessing considerable molecular diversity. The purification of a rat brain GABA transporter protein (*4*) provided a reagent for the molecular cloning of a GABA transporter cDNA. We report the isolation and sequence of such a cDNA clone and its functional characterization in *Xenopus* oocytes.

Rat brain GABA transporter protein, purified as described (*4*), was subjected to cyanogen bromide degradation, and several of the resulting fragments were sequenced (*5*). The sequence of the longest peptide (QPSEDIVRPENG) (*6*) was used to design oligonucleotide probes. Because sucrose density gradient RNA fractionation had shown that GABA transporter mRNA was in the 4- to 5-kb size range (*7*), a λ-ZAPII rat brain cDNA library containing inserts of 4 kb and greater (*8*) was screened with conventional plaque hybridization techniques. Two plaques were positive through successive platings. The mRNA was synthesized in vitro from each of these clones and was tested for its ability to express functional GABA transporters in *Xenopus* oocytes. One clone, which tested positive in the oocyte assay, was selected for detailed characterization and designated GAT-1 (GABA transporter 1).

Oocytes injected with GAT-1 RNA accumulated 50 to 100 times as much [^{3}H]GABA as water-injected or uninjected control oocytes (*9*) (Fig. 1A). In contrast, oocytes injected with 50 ng of rat brain polyadenylated RNA accumulated only 10 to 15 times as much [^{3}H]GABA as controls. Kinetic studies showed that GABA uptake by oocytes injected with GAT-1 mRNA is saturable (Fig. 1B), indicating the expression of a carrier-mediated uptake system. Eadie-Hofstee plots of three experiments revealed a Michaelis constant (K_m) of 3.1 to 10.6 μM (mean, 7.0 μM), which is

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within the range of values reported for high-affinity GABA uptake systems in the brain (*2*). The maximum velocity of uptake (V_{max}) varied considerably, from 3 to 26 pmol per oocyte per hour; this is not unexpected, given the variability of foreign RNA expression among different batches of oocytes.

The transporter encoded by GAT-1 has an absolute requirement for extracellular Na^+ ions. Replacement of sodium chloride with either lithium chloride or choline chloride reduced transport to background levels (Fig. 1C). The GABA transporter encoded by this clone is also Cl^--dependent; replacement of Cl^- ions with acetate abolished uptake, and replacement by nitrate decreased uptake by 64 to 83% (Fig. 1C). The ability of nitrate ions to substitute partially for Cl^- agrees with the results of GABA uptake assays with rat brain membrane vesicles (*10*).

The pharmacological sensitivity of GAT-1 was tested with five well-characterized GABA uptake blockers (Fig. 1D): nipecotic acid (NIP), a potent, nonselective blocker (*11*); *cis*-3-aminocyclohexane carboxylic acid (ACHC) and 2,4-diaminobutyric acid (DABA), which are selective for neuronal uptake (*12*); and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO) and β-alanine (β-Ala), which are selective for glial uptake (*13, 14*). NIP was the most effective inhibitor, reducing uptake by about 50% at 10 μM. ACHC and DABA were less potent than NIP, each resulting in about 50% inhibition at 100 μM. THPO and β-Ala were the least effective of the blockers, each resulting in about 20 to 30% inhibition at 100 μM. We have not yet determined whether any of these drugs act as substrates for this transporter.

The protein encoded by GAT-1 cross-reacted with an antiserum (*15*) against the native rat brain GABA transporter (Fig. 2). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of immunoprecipitated samples (*16*) showed a major band of 67 kD, similar to the estimated molecular weight of the GABA transporter core protein (*15*). This band was absent in samples without mRNA template or samples treated with control serum. Thus, the GAT-1 protein shares antigenic determinants with the native rat brain GABA transporter.

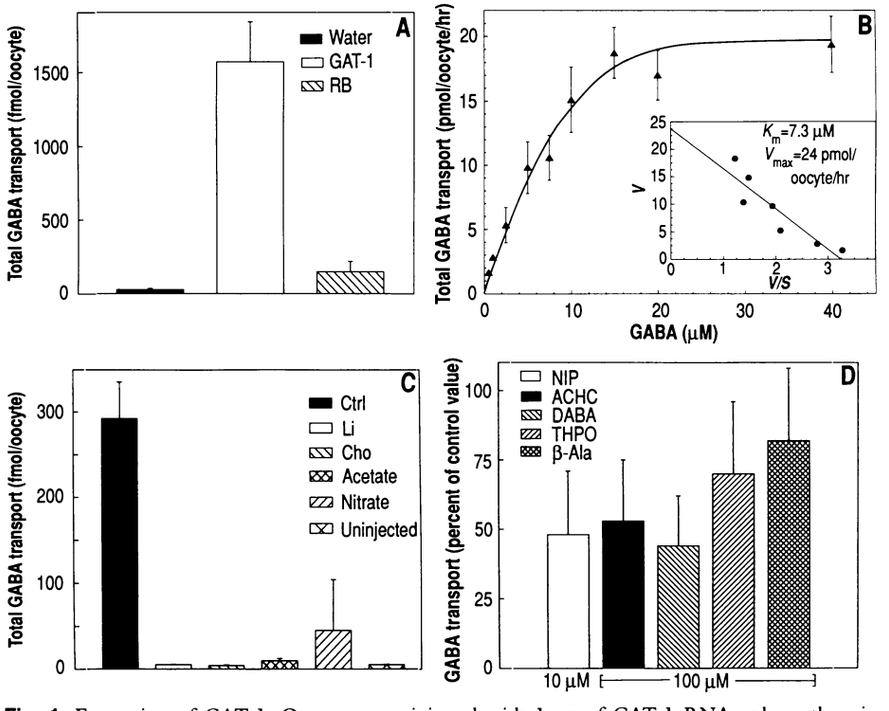

Fig. 1. Expression of GAT-1. Oocytes were injected with 1 ng of GAT-1 RNA unless otherwise indicated. Background values are [^{3}H]GABA uptake by water-injected or uninjected control oocytes. Each value is the mean ± SEM of four or five samples. The length of incubation with [^{3}H]GABA was 60 min. (**A**) GABA transporter expression induced by 50 ng of rat brain polyadenylated RNA (RB) and by 10 ng of GAT-1 RNA (GAT-1). (**B**) Transporter kinetics. [^{3}H]GABA accumulation was measured with GABA concentrations from 0.5 μM to 40 μM. The values in the saturation curve represent total GABA uptake; uptake by uninjected oocytes (representing the nonspecific component) comprised 2 to 6% of the total. (Inset) Eadie-Hofstee plot obtained with substrate concentrations from 0.5 μM to 15 μM. The data points are the difference between uptake values measured in injected and uninjected oocytes. [*V*, GABA uptake (picomoles per oocyte per hour); *S*, free substrate concentration.] (**C**) Effect of different incubation media on total GABA transport. Oocytes were assayed in ND96 (Ctrl) or modifications of this saline. Li, 96 mM LiCl; Cho, 96 mM choline chloride. (**D**) Effect of GABA uptake blockers on specific GABA transport. Oocytes were incubated in ND96 for 15 min in the absence of drugs. Drugs were then present during a 60-min incubation period at a concentration of 100 μM, except for NIP which was at 10 μM. Data are expressed as a percentage of uptake seen in the absence of any drugs.

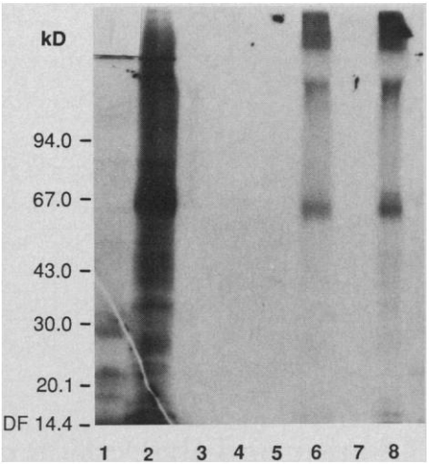

Fig. 2. The GAT-1 protein cross-reacts with an antiserum to the GABA transporter. GAT-1 RNA was translated in vitro and incubated with antisera, and the translation products were analyzed by SDS-PAGE (*16*). The exposure of this photograph was optimized to show the 67-kD bands in lanes 6 and 8; in shorter exposures the difference in density between the 67-kD band in lane 2 and background staining is greater. Lanes 1 and 2, total translation mixtures (no immunoprecipitation) incubated without GAT-1 mRNA template (lane 1) and with 1 μg of GAT-1 mRNA template (lane 2); lanes 3 through 8, pellets from immunoprecipitated lysates under the following conditions: lane 3, no GAT-1 mRNA template, control antiserum; lane 4, no GAT-1 mRNA template, antiserum to the GABA transporter; lane 5, 1 μg of GAT-1 mRNA template, control serum with Triton X-100; lane 6, 1 μg of GAT-1 mRNA template, antiserum to the GABA transporter with Triton X-100; lane 7, 1 μg of GAT-1 mRNA template, control serum, no Triton X-100; lane 8, 1 μg of GAT-1 mRNA template, antiserum to the GABA transporter with no Triton X-100. DF, dye front.

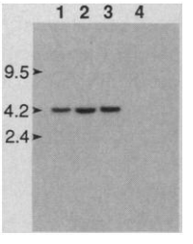

Fig. 3. GAT-1 labels a 4.2-kb brain transcript. Rat brain polyadenylated RNA (4.5 μg) was separated by electrophoresis through a 1% agarose-formaldehyde minigel, transferred to Immobilon-N, and hybridized to the nick-translated Hind III–Apa I fragment of GAT-1. Hybridization conditions were 50% formamide, 1.08 M NaCl, 0.06 M Na_3PO_4, 0.006 M EDTA (6× SSPE), 5× Denhardt's, 0.5% SDS, salmon sperm DNA (100 μg/ml) at 42°C for 20 hours. RNA size standards, run in parallel lanes, were stained with ethidium bromide. Lane 1, cerebrum; lane 2, cerebellum; lane 3, brainstem; lane 4, liver. Numbers in left margin represent kilobases.

We conclude that GAT-1 encodes a high-affinity, Na^+- and Cl^--dependent, plasma membrane GABA transporter with a drug sensitivity similar to the neuronal subtype. The protein encoded by GAT-1 shares antigenic sites with the native GABA transporter. The fact that a single clone can express GABA uptake activity suggests that the GABA transporter functions as a single subunit or as a multimer of identical subunits.

We examined GAT-1 expression in rat brain by probing polyadenylated RNA from cerebrum, cerebellum, and brainstem with nick-translated GAT-1 (Fig. 3). A single band of about 4.2 kb was visualized in each brain sample, a size that agrees with the results of RNA fractionation experiments (*7*); no bands were detectable in liver mRNA. This result contrasts with reports that rat cerebellar mRNA contains GABA transporter transcripts in the 2.0- to 3.0-kb size range (*17*). The reason for this discrepancy is not known.

The nucleotide sequence of GAT-1 indicated that the clone contained an insert that was 4054 nucleotides long, with a predicted open reading frame of 1797 nucleotides, a 5′ untranslated region of 149 nucleotides, and a 3′ untranslated region of 2108 nucleotides (Fig. 4) (*18*). The region immediately surrounding the putative start codon contains purines at positions +4 and −3, and therefore represents a reasonable Kozak consensus sequence (*19*); furthermore, the proposed start codon is 89 bases downstream from a single in-frame stop codon. The open reading frame predicts a protein containing 599 amino acids with a molecular weight of 67 kD, which agrees with the molecular weight of the deglycosylated GABA transporter protein (*15*) and with the size of the in vitro translation product (Fig. 2). The sequence of the peptide used to design the oligonucleotide probes is present near the COOH-terminus of the deduced protein, as are the sequences of four other peptides isolated from the cyanogen bromide fragment mixture. The deduced protein contains four potential glycosylation sites (Fig. 4). One potential protein kinase A phosphorylation site and seven potential protein kinase C phosphorylation sites are present.

An analysis of the GAT-1 deduced protein suggests the presence of at least 11 to 13 transmembrane regions (Fig. 5A). This large number of membrane spanning domains is an emerging structural motif of transport proteins. The NH_2-terminal end of GAT-1 is only moderately hydrophobic and does not score as a signal sequence with von Heinje's algorithm (*20*). We have modeled GAT-1 secondary structure with 12 transmembrane regions (Fig. 5B). Because of the apparent absence of a signal sequence, the NH_2-terminal end of the protein has been placed in the cytoplasm. This orientation puts three of the four putative glycosylation sites on the extracellular face of the protein, which is consistent with the observed neuraminidase sensitivity of GABA uptake by synaptosomes (*21*). The transmembrane regions are portrayed as α helices that are 21 residues long; however, in multispanning membrane proteins, these regions could be shorter or could assume other secondary structures (*22*).

A search of the available databases did not uncover any significant sequence similarities between GAT-1 and other proteins (*18*). Furthermore, direct sequence comparisons of GAT-1 with the following proteins did not reveal any extended regions of homology: the Na^+-dependent glucose, proline, and branched-chain amino acid transporters (*23*); the $GABA_A$ and glycine receptors (*24*); the brain anion exchanger AE3 (*25*);

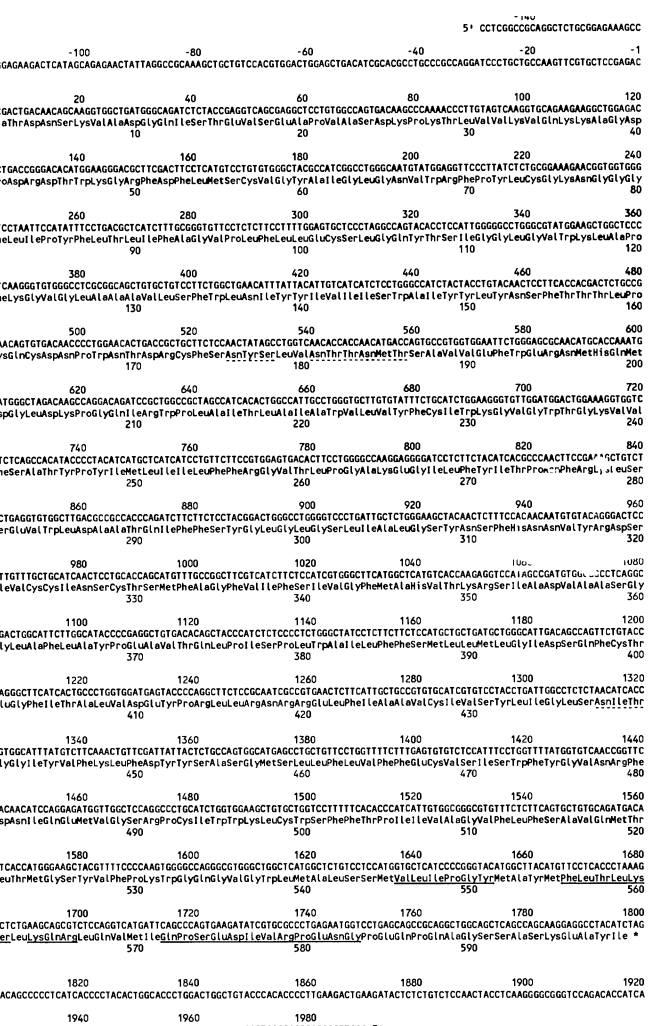

Fig. 4. Nucleotide sequence of GAT-1. Only a portion of the 3′ untranslated region is shown. Solid underline, peptides from the cyanogen bromide digest of the native GABA transporter protein; dashed underline, potential glycosylation sites. The entire sequence of GAT-1 has been deposited with Genbank (*27*).

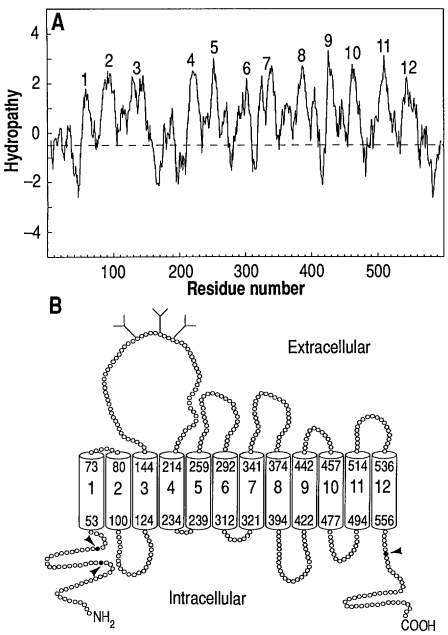

Fig. 5. Hydropathy plot and hypothetical secondary structure of the GAT-1 deduced protein. (**A**) Kyte-Doolittle analysis with a window of nine amino acids (*28*). Twelve putative transmembrane regions are numbered. (**B**) Schematic diagram of possible membrane orientation of GAT-1. Individual amino acid residues are shown as circles. Putative transmembrane segments are depicted as cylinders; the end points of these segments are numbered according to predictions from the method of Eisenberg and coworkers (*29*). Three putative protein kinase C phosphorylation sites (Ser^{24}, Thr^{46}, and Ser^{562}) are shown as black circles and marked with arrowheads. The remaining four protein kinase C sites and the single protein kinase A site are located externally or within membrane segments and are not illustrated. Three of the four putative glycosylation sites are located on the large extracellular loop connecting membrane segments 3 and 4 and are depicted as branched lines; the remaining putative glycosylation site is located within membrane segment 9 and is not shown.

and a Na^+-H^+antiporter (*26*). GAT-1 appears to be a member of a novel family of proteins.

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6. The single-letter amino acid codes are A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.
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9. [^{3}H]GABA uptake assays were performed on individual oocytes injected with 50 nl of various RNA solutions or water. The oocytes were incubated for 15 min at room temperature in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, buffered to pH 7.5 with 5 mM Hepes) containing 100 μM aminooxyacetic acid, and the transport reaction was initiated by the addition of [^{3}H]GABA (111 Ci/mmol; New England Nuclear) to a final concentration of 0.2 μM. After a 60-min incubation period, the oocytes were rapidly washed and solubilized in 10% SDS, and the radioactivity was measured by liquid scintillation counting. For the pharmacology experiments, drugs were present only during the incubation with [^{3}H]GABA. RNA was extracted from rat brain with the LiCl-urea method of C. Auffray and R. Rougeon [*Eur. J. Biochem.* **107**, 303 (1980)] or the guanidinium thiocyanate method of P. Chomczynski and N. Sacchi [*Anal. Biochem.* **162**, 156 (1987)]. For synthesis of GAT-1 RNA in vitro, the plasmid harboring GAT-1 was linearized with Not I and used as a template in transcription reactions that contained 1 mM each of adenosine triphosphate, cytidine triphosphate, and uridine triphosphate, 0.2 mM of guanosine triphosphate, 1 mM of 5′-(7-methyl)-guanosine-guanosine triphosphate, and 80 U of T7 RNA polymerase in a total volume of 100 μl. The reaction mixtures were incubated for 2 hours at 37°C and then treated with 40 U of deoxyribonuclease I for 30 min at 37°C. After two phenol extractions, the RNA was recovered by ethanol precipitation, and the free nucleotides were removed with a Sephadex G-50 spin column.
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