

# Molecular cloning of an orphan transporter

## A new member of the neurotransmitter transporter family

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**Abstract** A complementary DNA clone predicted to encode a novel transporter was isolated from rat brain and the localization of its mRNA was examined. The cDNA, designated rB21a, predicts a protein with 12 putative transmembrane domains that exhibits significant sequence homology with neurotransmitter transporters. Expression studies have not yet identified the endogenous substrate for this transporter, but the presence of rB21a mRNA within the leptomeninges of the brain suggests the transporter may regulate CSF levels of its substrate. The cloning of rB21a provides the means to determine its physiological functions and the potential to design novel, transporter-based therapeutic agents for neurological and psychiatric disorders.

**Key words:** Transporter; mRNA localization; Molecular cloning; Astrocyte; Brain (rat)

### 1. Introduction

Transporters throughout the body control the solute composition of the CSF, urine, plasma, and other extracellular fluids. In the brain, neurotransmitter transporters subserve specialized functions related to the modulation of synaptic transmission. The cloning of genes encoding neurotransmitter transporters is facilitating the elucidation of the role of transport proteins in nervous system function. The availability of cloned transporters provides the opportunity to define the pharmacological profiles of specific gene products, map their patterns of distribution, and make correlations with *in vivo* observations to better understand their biological functions.

Since the description of the primary structure of the first neurotransmitter transporter, the GABA transporter GAT-1 [1], at least 10 additional transporters with related sequences have been cloned (see [2] for review), defining a new gene family. Members of this family typically exhibit from ~40% to ~70% overall amino acid identity with each other, and share a number of structural and functional features, including 12 predicted transmembrane domains, potential sites for glycosylation in extracellular domains, and dependence on sodium for transport activity. Transporters in this family include those for

norepinephrine [3], serotonin [4,5], dopamine [6,7], glycine [8–12], GABA [1,13–15], betaine [16,17], taurine/ $\beta$ -alanine [18–20], L-proline [21], and creatine [22]. All of these transporters are found in the brain and their substrates typically serve as neuroregulators, osmoregulators, or both, reinforcing the concept that molecules with similar structures often have similar functions.

Several additional transporters have been cloned that exhibit significant amino acid identity with the neurotransmitter transporters, but whose endogenous substrates have not yet been identified [23–25]. We describe here the cloning and localization of a novel ‘orphan’ transporter from rat brain, designated rB21a, whose structural homology with neurotransmitter transporters indicates it is related to this gene family. Its presence in restricted brain areas and cell types further suggests that its endogenous substrate may be neuroactive. As a novel transporter homologous to neurotransmitter transporters, rB21a may be useful as a target for the development of therapeutic agents for neurological and psychiatric disorders. The cloning of rB21a provides the basis for exploring its functions in the nervous system and elsewhere.

### 2. Experimental

#### 2.1. Screening of the rat brain cDNA library and sequencing

A rat brain cDNA library in the Lambda ZAP II vector (Stratagene, La Jolla, CA) was screened at low stringency as previously described [8] using probes representing the coding region of the rat GAT-1 GABA transporter cDNA [1]. Lambda phage hybridizing to the probe were plaque purified and screened with the same probe mixture at high stringency to eliminate GAT-1 cDNAs. rB21a clones were converted to phagemids by *in vivo* excision with f1 helper phage. Nucleotide sequences of cDNAs in pBluescript were analyzed on both strands by the Sanger dideoxy nucleotide chain-termination method [26] using Sequenase (U.S. Biochemical Corp., Cleveland, OH).

#### 2.2. Expression and uptake assays in COS-7 cells

Two cDNA clones were identified which collectively span the entire coding region of the rB21a transporter gene, including 274 base pairs of 5′ untranslated sequence and 902 base pairs of 3′ untranslated sequence. The two clones were ligated at their internal *Bam*HI sites to create a full-length 3.0 kb cDNA and subcloned into the eukaryotic expression vector pcEXV-3 [27] for pharmacological characterization. Transient transfection of COS-7 cells with rB21a was carried out using DEAE-dextran with DMSO according to the method of Lopata et al. [28] with minor modifications. Transport of putative substrates was measured in COS-7 cells two days after transfection as previously described [13] using 50 nM [<sup>3</sup>H]substrate (6–90 Ci/mmol; New England Nuclear, Boston, MA). Non-specific uptake was defined in parallel wells with 1 mM unlabeled substrate.

#### 2.3. Preparation of cell cultures

Primary cultures of neurons, astrocytes, and fibroblasts were prepared from E19 embryonic rats. Briefly, the brains were removed,

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**Abbreviations:** GABA,  $\gamma$ -aminobutyric acid; CSF, cerebrospinal fluid; TM, transmembrane domain; RT-PCR, reverse transcriptase-polymerase chain reaction.

dissected free of meninges, and trypsinized. Cells were dissociated mechanically by passage through a Pasteur pipette, and resuspended in DMEM containing 10% fetal bovine serum and antibiotics. The cells were added to tissue culture dishes that had been previously coated with 10  $\mu$ M poly-D-lysine. Type 1 and O-2A/Type 2 astrocyte cultures were prepared as described elsewhere [29]. For neurons, a plating density of  $15 \times 10^6$  cells per 100 mm dish was employed; the medium was supplemented with insulin. Cytosine arabinoside was added to a final concentration of 10  $\mu$ M on day 2 or 3 to inhibit the proliferation of non-neuronal cells; the neurons were harvested 1 week after plating. To obtain meningeal cells, the meninges were trypsinized and mechanically dissociated as described above. The cells recovered from a single embryo were plated into a 100 mm dish, grown to confluence, and passaged 1–2 times prior to harvesting. Fibroblasts from skin were prepared as described for meningeal cells.

#### 2.4. Northern blot analysis

Total cellular RNA was isolated from rat tissues and cells using guanidine isothiocyanate and collected by centrifugation through cesium chloride [30], as modified from the method of Chirgwin et al. [31]. Poly(A)<sup>+</sup> RNAs were purchased from Clontech (Palo Alto, CA). Denatured RNA samples (5–25  $\mu$ g) were separated by electrophoresis in 1.0% agarose gels containing 2.7% formaldehyde. RNAs were transferred to GeneScreen Plus membranes (Dupont-NEN, Boston, MA) by overnight capillary blotting in 10 $\times$  SSC. Northern blots were rinsed and then baked for 2 h at 80°C under vacuum. Prehybridization was for 1–2 h at 42°C in a solution containing 50% formamide, 1 M sodium chloride, 10% dextran sulfate and 1.0% SDS. Blots were hybridized overnight at 42°C with <sup>32</sup>P-labeled rB21a cDNA (randomly primed) in prehybridization mixture containing 100  $\mu$ g/ml sonicated salmon sperm DNA. The blots were washed successively in 2 $\times$  SSC/2% SDS, 1 $\times$  SSC/2% SDS, and 0.2 $\times$  SSC/2% SDS at 65°C, then exposed to Kodak XAR-5 film with one intensifying screen at –80°C for up to three weeks. After hybridization with rB21a, blots were routinely reprobbed with 1B15 [32] in order to confirm that equal amounts of RNA were present in each lane.

#### 2.5. In situ hybridization

Male Sprague–Dawley rats (150–250 g) were decapitated and their brains and peripheral tissues were rapidly removed and frozen by immersion in isopentane on dry ice. Tissues were sectioned at 11  $\mu$ m on a cryostat, thaw-mounted onto poly-L-lysine-coated slides, and stored at –20°C until use. Pairs of antisense and sense oligonucleotides designed to the 7/8 loop and the 3'-untranslated region were synthesized on a Cyclone Plus DNA Synthesizer (Milligen/Bioresearch). The sequences of the oligonucleotides (45-mers) were:

Sense, 7/8 loop: 5'-CTGGAGGAGGTGAAGGACTACCTGGCA-TCTACTTACCAAACAAG

Antisense, 7/8 loop: 5'-CTTGTTTGGGTAAGTAGATGCCAGG-TAGTCCTTCACCTCCTCAG

Sense, 3'UT: 5'-GTCACCTTCTCTGAATCTAAGGTTCTCA-CAGTGGGCCAGGACA

Antisense, 3'UT: 5'-TGTCCTGGCCCACTGTGAGGAACCTTA-GATTCAGAGAAAGGTAC

Probes were 3'-end labeled with [<sup>35</sup>S]dATP (1,200 Ci/mmol, New England Nuclear, Boston, MA) to a specific activity of 10<sup>9</sup> dpm/ $\mu$ g using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN). Hybridization buffer consisted of 50% formamide, 4 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1 $\times$  Denhardt's solution (0.2% polyvinyl-pyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin), 50 mM dithiothreitol, 0.5 mg/ml yeast tRNA, and 10% dextran sulfate. Radiolabeled probe in 100  $\mu$ l hybridization buffer was applied to each tissue section and hybridized overnight at 50°C. Sections were washed for 2 $\times$  30 min in 2 $\times$  SSC at room temperature, for 1 h in 1 $\times$  SSC at room temperature, for 1 h in 2 $\times$  SSC at 50°C, and for 30 min in 0.1 $\times$  SSC at room temperature. Tissues were dehydrated in a series of graded alcohols, apposed to Kodak XAR-5 film for two weeks, then dipped in Kodak NTB3 autoradiography emulsion diluted 1:1 with 0.2% glycerol. After four weeks, slides were developed and counterstained with hematoxylin and eosin, and the cellular distribution of the exposed silver grains was examined with a Zeiss Axioskop. Parallel tissues pretreated with 100  $\mu$ g/ml RNase A (37°C, 30 min) prior to hybridization showed no significant signal. Both

pairs of oligonucleotides for rB21a (see above) showed identical patterns of hybridization.

### 3. Results

#### 3.1. Cloning

To clone novel neurotransmitter transporters we screened a rat brain cDNA library at low stringency with probes encoding the rat GABA transporter GAT-1 [1]. A clone was identified, rB21a, that hybridized at low but not at high stringency with the GABA transporter probes, indicating it was related but not identical to GAT-1. DNA sequence analysis revealed that the clone encoded a putative transporter with 53% nucleotide identity to GAT-1. Searches of GenBank and EMBL data bases demonstrated that the nucleotide sequence of rB21a was novel and that the most closely related sequence was that of an orphan transporter designated NTT4 [24].

rB21a, a full-length cDNA, contained a 3.0 kb sequence with an open reading frame of 1,848 base pairs which could encode a protein of 616 amino acids with a relative molecular mass of ~67,000 Daltons (Fig. 1). The deduced amino acid sequence of rB21a exhibited significant homology (36–41% amino acid identity) with the neurotransmitter transporter gene family (Table 1). Slightly higher amino acid identities were observed with the 'orphan' transporters NTT4 [24] and v7-3-2 [23] (~43%), while unrelated transporters such as the sodium/glucose cotransporter [33] exhibited much lower identity (~17%). Hydropathy analyses of rB21a indicate the presence of twelve putative transmembrane domains (TMs); potential sites for Asn-linked glycosylation are located in the predicted second and fourth extracellular loops based on the membrane topology proposed for GAT-1 [1]. Alignment of rB21a with the GAT-2 and glycine transporters (Fig. 2) reveals that despite regions of high amino acid sequence conservation, rB21a is predicted to have a significantly longer loop between TMs VII and VIII than previously cloned neurotransmitter transporters.

#### 3.2. Pharmacology

Sequence comparisons suggested that rB21a might encode a neurotransmitter transporter. To explore this possibility, a cDNA containing the full coding region of the rB21a transporter was placed in a mammalian expression vector (pEXV-3), transfected into COS-7 cells, and screened for transport of a variety of radiolabeled neurotransmitters and amino acids. COS-7 cells transiently transfected with rB21a (COS/rB21a) failed to accumulate the potential substrates to a greater extent than non-transfected control cells (Table 2). The substrates tested included inhibitory amino acid neurotransmitters, monoamine neurotransmitters, amino acids and substrates for amino acid transporters, and other related endogenous compounds. Thus, the rB21a cDNA does not appear to encode a transporter for commonly known classical neurotransmitters.

#### 3.3. Localization

To gain insight into the potential function of the rB21a transporter, we carried out localization studies of the mRNA encoding the transporter. Northern blot analysis of poly(A)<sup>+</sup> RNAs from various rat tissues revealed a 3.0 kb transcript in brain, kidney, and lung that hybridized at high stringency with rB21a (Fig. 3). In the kidney, an additional ~2.6 kb hybridizing transcript was present which could represent either a related

GGCAGGGTGGGGCTCACCGCTCCCCACTTACCGGCTCGCCCTCTCGTGCGCGTTA	ATG AGA TTA GCA ATT AAA	18
	Met Arg Leu Ala Ile Lys	6
AGG CGG GCG AGC CGC GGC CAG AGA CCA GGC CCT GAC GAG AAG CGA GCG CGG GAC ATG GAG AAG GCA CGG CCT CAA		93
Arg Arg Ala Ser Arg Gly Gln Arg Pro Gly Pro Asp Glu Lys Arg Ala Arg Asp Met Glu Lys Ala Arg Pro Gln		31
TGG GGC AAT CCG CTG CAG TTC GTT TTC GCC TGT ATC TCC TAC GCC GTG GGT TTG GGC AAT GTG TGG CGC TTC CCC		168
Trp Gly Asn Pro Leu Gln Phe Val Phe Ala Cys Ile Ser Tyr Ala Val Gly Leu Gly Asn Val Trp Arg Phe Pro		56
TAC CTG TGC CAG ATG TAC GGC GGA GGG AGT TTC CTG GTC CCC TAC CTC ATC ATG CTC ATT GTG GAG GGG ATG CCA		243
Tyr Leu Cys Gln Met Tyr Gly Gly Gly Ser Phe Leu Val Pro Tyr Leu Ile Met Leu Ile Val Glu Gly Met Pro		81
CTC TTG TAC CTG GAG CTG GCT GTG GGG CAG CGC ATG CGG CAG GGC AGC ATT GGT GCC TGG AGG ACC ATC AGC CCC		318
Leu Leu Tyr Leu Glu Leu Ala Val Gly Gln Arg Met Arg Gln Gly Ser Ile Gly Ala Trp Arg Thr Ile Ser Pro		106
TAC CTT AGT GGT GTC GGT GTT GCC AGT GTG GTG GTC TCC TTC TTC CTC TCC ATG TAC TAC AAT GTC ATC AAT GCC		393
Tyr Leu Ser Gly Val Gly Val Ala Ser Val Val Val Ser Phe Phe Leu Ser Met Tyr Tyr Asn Val Ile Asn Ala		131
TGG GGC TTC TGG TAC CTC TTC CAC TCC TTC CAG GAT CCC CTG CCG TGG TCT GTC TGC CCA CTG AAT AGT AAC CGC		468
Trp Gly Phe Trp Tyr Leu Phe His Ser Phe Gln Asp Pro Leu Pro Trp Ser Val Cys Pro Leu Asn Ser Asn Arg		156
ACA GGC TAT GAT GAG GAG TGT GAG AAG GCT TCG TCG ACA CAG TAC TTC TGG TAC AGG AAA ACA CTC AAC ATC TCA		543
Thr Gly Tyr Asp Glu Glu Cys Glu Lys Ala Ser Ser Thr Gln Tyr Phe Trp Tyr Arg Lys Thr Leu Asn Ile Ser		181
CCG TCC ATC CAG GAG AAT GGA GGA GTG CAG TGG GAG CCA GCC CTG TGC CTC ACC CTG GCC TGG CTG ATG GTA TAT		618
Pro Ser Ile Gln Glu Asn Gly Gly Val Gln Trp Glu Pro Ala Leu Cys Leu Thr Leu Ala Trp Leu Met Val Tyr		206
CTG TGC ATC CTG AGA GGC ACC GAA TCT ACT GGC AAG GTG GTC TAC TTC ACC GCA TTG ATG CCT TAC TGT GTT CTT		693
Leu Cys Ile Leu Arg Gly Thr Glu Ser Thr Gly Lys Val Val Tyr Phe Thr Ala Leu Met Pro Tyr Cys Val Leu		231
ATT ATC TAC TTG GTC CGT GGC CTC ACA CTC CAT GGA GCC ACC AAT GGC CTG ATG TAC ATG TTC ACA CCT AAG ATT		768
Ile Ile Tyr Leu Val Arg Gly Leu Thr Leu His Gly Ala Thr Asn Gly Leu Met Tyr Met Phe Thr Pro Lys Ile		256
GAG CAG CTA GCC AAC CCC AAG GCC TGG ATC AAT GCA GCC ACG CAG ATC TTC TTC TCA CTG GGC TTG GGT TTT GGC		843
Glu Gln Leu Ala Asn Pro Lys Ala Trp Ile Asn Ala Ala Thr Gln Ile Phe Phe Ser Leu Gly Leu Gly Phe Gly		281
AGC CTG ATC GCT TTT GCC AGC TAC AAT GAA CCC TCC AAC GAC TGC CAG AAG CAT GCT GTC ATT GTG TCT GTC ATC		918
Ser Leu Ile Ala Phe Ala Ser Tyr Asn Glu Pro Ser Asn Asp Cys Gln Lys His Ala Val Ile Val Ser Val Ile		306
AAC AGC TCC ACC TCC ATA TTT GCC AGC ATT GTC ACC TTC TCC ATC TAT GGC TTC AAG GCC ACC TTC AAC TAT GAA		993
Asn Ser Ser Thr Ser Ile Phe Ala Ser Ile Val Thr Phe Ser Ile Tyr Gly Phe Lys Ala Thr Phe Asn Tyr Glu		331
AAC TGC TTA AAC AAG GTG ATT CTG CTG CTG ACC AAT TCT TTT GAC CTT GAA GAT GGC TTT CTG ACA GCC AGC AAC		1068
Asn Cys Leu Asn Lys Val Ile Leu Leu Leu Thr Asn Ser Phe Asp Leu Glu Asp Gly Phe Leu Thr Ala Ser Asn		356
CTG GAG GAG GTG AAG GAC TAC CTG GCA TCT ACT TAC CCA AAC AAG TAC AGT GAA GTG TTC CCA CAC ATT AGA AAC		1143
Leu Glu Glu Val Lys Asp Tyr Leu Ala Ser Thr Tyr Pro Asn Lys Tyr Ser Glu Val Phe Pro His Ile Arg Asn		381
TGC AGC TTG GAA TCA GAG CTG AAC ACG GCT GTC CAA GGC ACA GGC CTG GCC TTC ATC GTC TAC GCT GAG GCC ATT		1218
Cys Ser Leu Glu Ser Glu Leu Asn Thr Ala Val Gln Gly Thr Gly Leu Ala Phe Ile Val Tyr Ala Glu Ala Ile		406
AAA AAC ATG GAA GTG TCC CAG CTC TGG TCA GTG CTC TAC TTC TTC ATG CTG CTG ATG CTG GGA ATG GGG AGC ATG		1293
Lys Asn Met Glu Val Ser Gln Leu Trp Ser Val Leu Tyr Phe Phe Met Leu Leu Met Leu Gly Met Gly Ser Met		431
CTT GGA AAT ACA GCG GCC ATC CTC ACC CCT CTG ACT GAC AGC AAG GTC ATC TCC AGC TAC CTG CCC AAG GAG GCC		1368
Leu Gly Asn Thr Ala Ala Ile Leu Thr Pro Leu Thr Asp Ser Lys Val Ile Ser Ser Tyr Leu Pro Lys Glu Ala		456
ATT TCA GGT CTG GTG TGC CTC ATT AAC TGT GCT GTT GGC ATG GTG TTC ACC ATG GAG GCT GGG AAC TAC TGG TTT		1443
Ile Ser Gly Leu Val Cys Leu Ile Asn Cys Ala Val Gly Met Val Phe Thr Met Glu Ala Gly Asn Tyr Trp Phe		481
GAC ATA TTC AAT GAC TAT GCA GCC ACG CTG TCT CTG CTG CTC ATT GTG CTG GTG GAG ACT ATA GCT GTG TGC TAC		1518
Asp Ile Phe Asn Asp Tyr Ala Ala Thr Leu Ser Leu Leu Leu Ile Val Leu Val Glu Thr Ile Ala Val Cys Tyr		506
GTG TAT GGG CTG AGG AGA TTT GAA AGT GAT CTT CGG GCC ATG ACT GGC CGG CCC CTC AAC TGG TAC TGG AAG GCC		1593
Val Tyr Gly Leu Arg Arg Phe Glu Ser Asp Leu Arg Ala Met Thr Gly Arg Pro Leu Asn Trp Tyr Trp Lys Ala		531
ATG TGG GCT TTT GTG AGC CCA CTG CTC ATC ATC GGC CTC TTT ATC TTC TAC CTG AGT GAC TAC ATC CTC ACG GGA		1668
Met Trp Ala Phe Val Ser Pro Leu Leu Ile Ile Gly Leu Phe Ile Phe Tyr Leu Ser Asp Tyr Ile Leu Thr Gly		556
ACG CTG CAG TAC CAA GCC TGG GAT GCT ACT CAG GGG CAG CTG GTG ACC AAG GAT TAC CCT CCA CAT GCA CTA GCT		1743
Thr Leu Gln Tyr Gln Ala Trp Asp Ala Thr Gln Gly Gln Leu Val Thr Lys Asp Tyr Pro Pro His Ala Leu Ala		581
GTC ATC GGT TTG CTG GTG GCT TCA TCT ACT ATG TGC ATC CCC CTG GTG GCC CTG GGG ACT TTC ATC AGG AAT CGC		1818
Val Ile Gly Leu Leu Val Ala Ser Ser Thr Met Cys Ile Pro Leu Val Ala Leu Gly Thr Phe Ile Arg Asn Arg		606
CTC AAG AGG GGA GGC TCT TCC CCA GTG GCC TAAGAATGGACCTCCCAAAGACCGAAGTCAGCCACTCTGTTTCAC		1848
Leu Lys Arg Gly Gly Ser Ser Pro Val Ala End		616

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the transporter encoded by rB21a. Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown using three-letter symbols; potential sites for N-linked glycosylation are designated with arrowheads.

mRNA (transporter subtype) expressed only in kidney, or tissue-specific expression of an alternatively spliced transcript of rB21a. Quantitation of rB21a mRNA relative to 1B15, the mRNA encoding cyclophilin (data not shown), confirmed that the highest levels of rB21a mRNA were present in the brain, with lower levels in kidney and lung.

To complement and extend the Northern analyses of rB21a mRNA, in situ hybridization studies were carried out in sections of rat brain and kidney. As shown in Fig. 4, the mRNA encoding rB21a is abundant in the leptomeninges (pia/arachnoid) but is not observed elsewhere in the brain. In the kidney (Fig. 5), in agreement with Northern blot analysis, the tran-

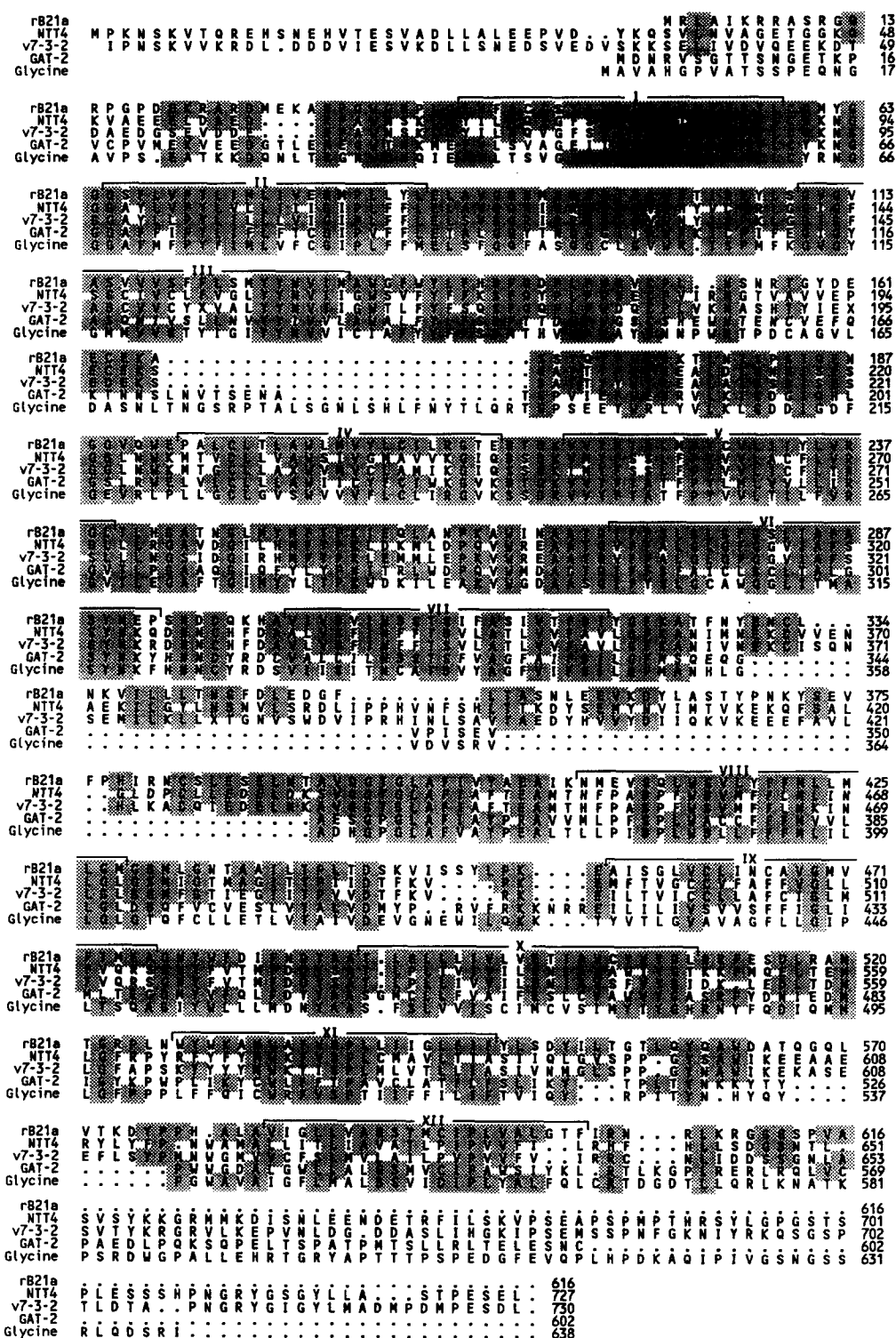


Fig. 2. Alignment of deduced amino acid sequence of rB21a with related transporters. Putative  $\alpha$ -helical membrane spanning domains (I-XII) are bracketed; residues identical to those of rB21a are shaded. NTT4 represents a rat brain 'orphan' transporter [24]; v7-3-2 represents an 'orphan' transporter cDNA isolated from rat midbrain [23]; GAT-2 represents the rat GAT-2 GABA transporter [13]; Glycine represents the rat glycine transporter [8].

script is less abundant than in brain; its pattern of distribution is limited to the outer medullary layer, which contains proximal convoluted tubules as well as ascending and descending limbs of the loop of Henle.

To ascertain which cell types in the brain express each transporter, we examined the cellular distribution of rB21a mRNA by Northern blot analysis of total RNA isolated from cultured neurons, Type 1 and O-2A/Type 2 astrocytes, leptomeningeal

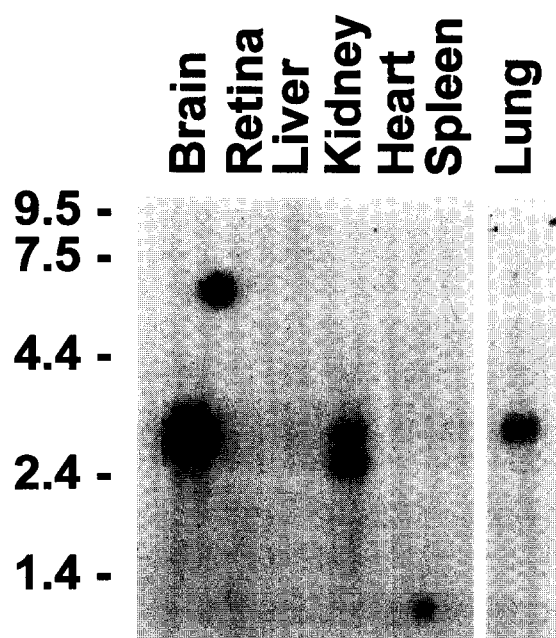


Fig. 3. Tissue Localization of rB21a by Northern blot analysis. Poly(A)<sup>+</sup> RNA (5 µg) from rat tissues was separated by formaldehyde/agarose gel electrophoresis, blotted to nylon membranes, and hybridized at high stringency with <sup>32</sup>P-labeled rB21a transporter cDNA. The locations of RNA size markers are indicated in kilobases. The hybridizing transcripts are ~3 kb and ~2.6 kb; the autoradiogram was exposed for 22 days. Rehybridization of the blot with 1B15 [32] revealed that retina contained less mRNA than the other lanes.

cells, and skin fibroblasts (Fig. 6). Interestingly, rB21a was predominantly expressed in O-2A/Type 2 astrocytes and leptomeningeal cells, with no detectable signal in neurons or skin fibroblasts; a very faint signal was seen in Type 1 astrocytes. RT-PCR amplification of rB21a cDNA from cultured neurons, astrocytes, and meningeal cells confirmed a nonneuronal pattern of expression (data not shown).

#### 4. Discussion

We have cloned a novel rat brain cDNA, designated rB21a,

Table 1  
Amino acid identity of rB21a with neurotransmitter and other transporters

Transporter	Amino acid identity (percent)
NTT4 [24]	43.6
v7-3-2 [23]	42.6
Betaine/GABA (BGT-1) [16]	40.5
Creatine [22]	40.3
Glycine [8]	40.1
GABA (GAT-2) [13]	39.8
Taurine [19]	39.5
GABA (GAT-3) [13]	38.6
GABA (GAT-1) [1]	38.3
L-Proline [21]	36.8
Norepinephrine [3]	36.7
Dopamine [6,7]	36.1
Serotonin [5]	36.4
Sodium/glucose [33]	17.3

Transporters represent rat sequences except betaine (dog), norepinephrine (human), and sodium/glucose (human).

that encodes a protein with significant homology to the recently described family of sodium-dependent neurotransmitter transporters. The degree of amino acid identity is similar to that observed between neurotransmitter transporters with distinct substrate selectivities (i.e. glycine transporter vs. taurine transporter, ~41%), but lower than between subtypes known to transport the same substrate (i.e. GAT-2 vs. GAT-3 GABA transporters, ~67%). The transporter encoded by rB21a is predicted to have a large, glycosylated loop between TMs VII and VIII, a feature not observed in previously cloned neurotransmitter transporters. Together with two orphan transporters recently cloned from rat brain which share this feature [23,24], rB21a may represent a structural subfamily within the transmitter transporter gene family.

We have not yet identified the endogenous substrate of the rB21a transporter. Despite significant sequence homology with

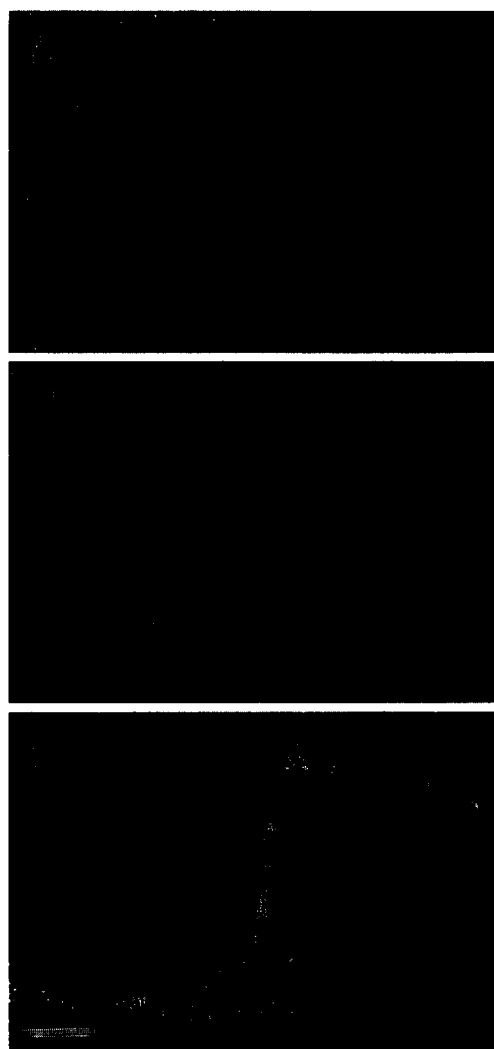


Fig. 4. Detection of rB21a transporter mRNA in the rat CNS by in situ hybridization. (A, B) Black and white reversal of film autoradiographs of coronal rat brain sections hybridized with <sup>35</sup>S-labeled (A) antisense oligonucleotide (7/8 loop) and (B) sense oligonucleotide, illustrating the transporter's localization in the leptomeninges. (C) Liquid emulsion autoradiograph of the rB21a transporter mRNA in leptomeningeal cells covering the ventral aspect of the brain. Note the lack of hybridization signal over smooth muscle cells of a blood vessel (asterisk). Bar = 100 µm.

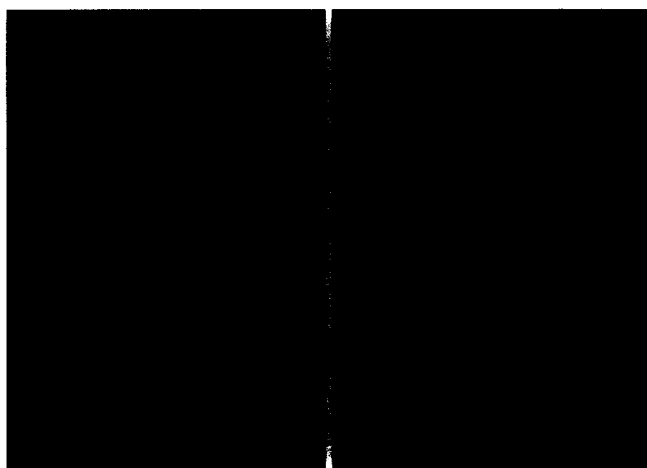


Fig. 5. Distribution of rB21a transporter mRNA in rat kidney. The hybridization observed with the rB21a antisense probe (A) is largely limited to the outer zone of the medulla (arrow); a low background is observed with the sense probe (B).

the family of neurotransmitter transporters, rB21a does not appear to encode a transporter for commonly known classical neurotransmitters. It is possible that the substrate represents a class of compounds unrelated to the modulation of synaptic transmission; alternatively, it has been suggested that identifying the substrates of 'orphan' transporters may reveal previously undescribed neurotransmitter systems [34]. Of the eleven transporters whose substrates have been characterized (see section 1), all are expressed in the brain and all but one (creatine transporter [22]) transport substrates believed to be neurotransmitters or neuromodulators. The presence of rB21a transporter mRNA in brain is consistent with a neuroactive role for its substrate.

Localization studies have provided clues as to the potential functions of rB21a. The distinct regional and cellular patterns of distribution of rB21a are suggestive of a specialized function for the transporter, in contrast with a generalized distribution expected of a ubiquitous metabolite transporter. The presence

of rB21a mRNA in the lung, kidney, and leptomeninges of the brain suggests a role in osmoregulation. Transporters in the pia could regulate the composition and volume of cerebrospinal fluid (CSF) and brain extracellular fluid, and could thereby play a role in homeostatic mechanisms to control brain swelling after trauma, hypoxia, seizures, or stroke. Pial transporters are situated to control the concentration of solutes such as neurotransmitters in the CSF; changes in levels of CSF neurotransmitters or metabolites can have profound effects on brainstem centers controlling functions such as respiration and blood pressure.

The detection of rB21a mRNA in cultured astrocytes suggests a wider distribution in the brain than the defined pattern revealed by *in situ* hybridization; a low level of expression per cell among large numbers of astrocytes may be detectable by Northern blot but not by *in situ* hybridization. Alternatively, astrocytes in culture may express higher levels of rB21a mRNA than astrocytes *in vivo*; it has been suggested that cultured O-2A/Type 2 astrocytes represent a class of reactive glial cells which proliferates in response to brain injury [35]. If rB21a mRNA in astrocytes is expressed under pathophysiological conditions, it may be difficult to detect in normal brain. Interestingly, the pattern of distribution of rB21a mRNA in the brain closely parallels that of the  $\beta$ -alanine sensitive GABA transporter GAT-2 [13], both in its meningeal localization [36] and its preferential expression in nonneuronal cells [29].

Table 2  
Potential substrates tested for transport at rB21a

Adenosine
Choline
Histamine
Glutamate
Tyrosine
GABA
Dopamine
Norepinephrine
Lysine
Serotonin
Taurine
Glycine
Melatonin
Alanine (for system ASC)
$\alpha$ -(Methylamino)isobutyric acid (for system A)

Cos-7 cells were transfected with rB21a and tested for transport of the substances shown, as described in section 2. None of the potential substrates exhibited increased transport in the transfected cells.

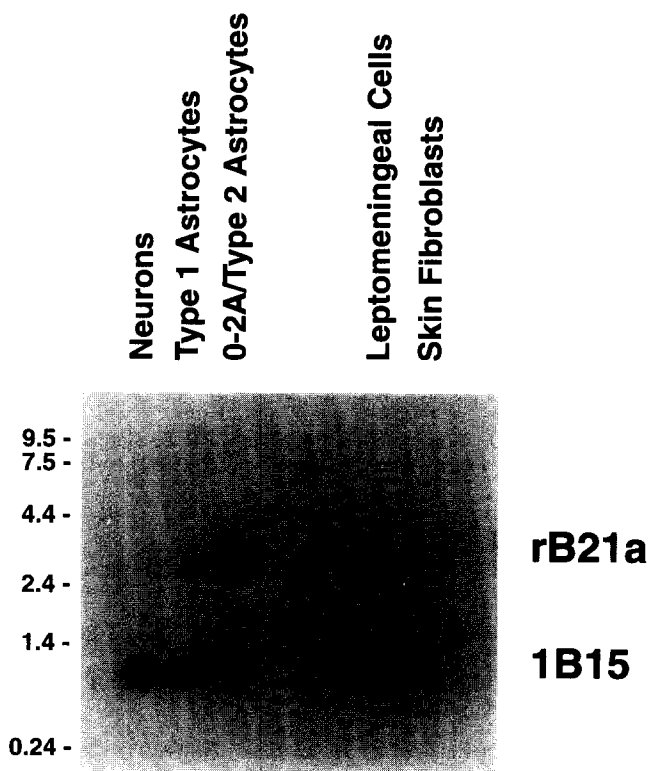


Fig. 6. Northern blot analysis of rB21a in primary cultures from rat brain. Northern blots of total RNA isolated from cultured neurons, astrocytes, meningeal cells, and skin fibroblasts (25  $\mu$ g) were prepared as described in Fig. 3. A ~3 kb hybridizing transcript is present in O-2A/Type 2 astrocytes and meningeal cells but not in neurons after a 5 day exposure of the autoradiogram. The blot was reprobed for 1B15 mRNA (~1 kb) to confirm that equal amounts of RNA were present in each lane.

When structure/function relationships within the neurotransmitter transporter gene family are better understood, it may be possible to predict interactions with substrates and inhibitors based on amino acid sequence alone. The cloning of the cDNA encoding rB21a has nevertheless provided the means to explore its physiological roles by pharmacological characterization, and by Northern and in situ mapping of its mRNA distribution. Further, the availability of the cDNA encoding the transporter will facilitate the development of antibodies and other reagents useful in defining the functions of the gene product in vivo. For example, antisense oligonucleotides which target mRNA molecules to selectively block translation of the gene product in vivo have been used successfully to relate the expression of a single gene with its functional sequelae [37,38]. The cloning of rB21a will allow the use of this approach to explore the functional consequences of blocking the expression of its mRNA without knowledge of its substrate.

In summary, we have isolated a rat brain cDNA encoding a novel 'orphan' transporter (rB21a) whose predicted amino acid sequence and restricted localization within the nervous system suggest functional as well as structural homology with neurotransmitter transporters. The highest levels of mRNA expression are in the leptomeninges of the brain, suggesting that the rB21a transporter may regulate CSF levels of its substrate. The cloning of this transporter provides the means to explore its physiological roles in the nervous system and elsewhere, and may thereby help to elucidate structure/function relationships within this expanding transporter gene family.

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