Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 419-427

THE CLONING AND EXPRESSION OF A HUMAN CREATINE TRANSPORTER

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Received August 25, 1994

A human creatine transporter (hCRT-BS2M) cDNA clone was isolated from a human brainstem/spinal cord using a PCR and phage plaque hybridization based technique. This clone included an open reading frame of 1,905 base pairs(bp) within a 2,283bp cDNA. Northern blot hybridization detected the expression of corresponding mRNAs most prominently in the skeletal muscle, heart and kidney. Peptide sequence analysis of the hCRT-BS2M protein product revealed 12 putative transmembrane domains. The predicted protein sequence further demonstrates that the hCRT-BS2M has highly conserved amino acid identity with the other members of the sodium dependent plasma membrane transporter family. Transient expression of the hCRT-BS2M in COS-7 cells demonstrates sodium dependent [^{14}C]creatine uptake with a K_M value of 14.9 ± 3.0 μM (n=5) that is attenuated by creatine and selective structural analogues of creatine.

Phosphocreatine is an important energy transducer in the heart, brain and skeletal muscle. In these tissues, creatine kinase catalyzes the reversible transfer of high-energy phosphate from adenosine triphosphate(ATP) to creatine to form creatine phosphate(1, 2). Cellular transport mechanisms of creatine are important for the metabolism of creatine in several tissues. The uptake system for creatine has been studied in a number of tissues and cell types, and revealed a sodium and chloride dependence(1-5). Complimentary DNAs encoding transporters for the gamma-amino butyric acid (GABA)(6-8), noradrenaline (NE) (9), dopamine (DA) (10-14), serotonin (5HT) (15-17), glycine (18, 19), proline (20) and taurine (21) have been cloned. They are all members of a growing family of plasma membrane bound transporters (22, 23). While trying to isolate a cDNA encoding a novel human choline transporter, we identified a transporter clone (hCRT-BS2M) present in a human brainstem/spinal cord cDNA library. Although the protein presented in this paper is related in sequence to the putative rat choline transporter (CHOT) (24), choline failed to be transported by this protein when expressed in cultured cells. During the characterization and expression of the hCRT-BS2M, the sequence of the rabbit creatine transporter (rbtCRT) was reported by Guimbal *et al.*(25). The high degree of homology between the hCRT-BS2M and the rabbit clone further

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Abbreviations: ratCRT, rat creatine transporter; hCRT-BS2M, human creatine transporter derived from the brainstem; rbtCRT, rabbit creatine transporter.

0006-291X/94 $5.00

confirms our hypothesis that we have cloned a human creatine transporter. Here, we report the structural and functional characterization of the human creatine transporter.

MATERIALS AND METHODS

Screening of the human brainstem/spinal cord cDNA library. In order to screen a human brainstem/spinal cord cDNA library (Stratagene), a probe was amplified using degenerate primers in a polymerase chain reaction (PCR). Primers were designed flanking a region of high homology between transmembranes (TMs) II and VI after examining the amino acid alignment of selected transporters (i.e. the NE, GABA, DA, 5HT, proline and glycine transporters). The primer sequences were as follows:
Sense, 5'-AACGGCGGAGGTG(T/C)(G/C)TTC(A/T/C)T(G/A/T/C)(G/A/T/C)T(G/T/C)CC(G/A/T/C)TA-3'
Antisense, 5'-GGCATAAGAGAAGAA(G/A/T/C)A(T/C)CTG(G/A)(G/A)(G/A/T/C)(A/T/C)CC(G/A/T)GC(G/A/T)TC-3'. PCR amplification conditions were as follows: 94°C for 1 min., 55°C for 2 min. and 72°C for 3 min. for 35 cycles. The expected 0.7 kb PCR product was obtained using the human brainstem/spinal cord cDNA λ ZAP library as a template. The PCR product was then subcloned into the TA vector, pCRII (Invitrogen). Sequencing the PCR product was achieved by the dideoxy method. A human brainstem/spinal cord cDNA λ ZAP library (Stratagene) was screened with the [^{32}P]dCTP labeled insert of the pCRII vector. Hybridization was carried out overnight at 65°C in a 0.05 M NaH_2PO_4, pH 7.4, buffer containing 5X Denhardt's solution, 6X SSC, salmon sperm DNA (0.01 mg/ml) and [^{32}P] labeled probe (1 X 10^7 cpm/ml). Filters were washed for 30 min. in 2X SSC/0.1% SDS buffer at room temperature, and then in 0.1X SSC/0.1% SDS buffer, at 65°C. Bluescript plasmid was rescued from purified positive λ phage clones by an *in vivo* excision method. Sequencing was achieved by the dideoxy method. The same screening procedures were performed on a human striatal cDNA library (Stratagene).

Northern Blot Analysis. The SacI-HindIII fragment corresponding to the downstream region of the hCRT-BS2M was radiolabeled with [^{32}P]dCTP and was hybridized to a Human Multiple Northern Tissue Blot (Clontech). The blot was hybridized in 5X SSPE, pH 7.4, 10X Denhardt's solution, 2% SDS and 100 μg/ml denatured salmon sperm for 18 hrs. at 65°C. The blot was washed with 2X SSC/0.05% SDS buffer at room temperature for 40 min., followed by two rinses at 50°C in 0.1X SSC/0.1% SDS buffer for 40 min. each, and then exposed to Hyperfilm MP (Amersham) for one week.

Transient Expression of cDNA and [^{14}C]Creatine Uptake in COS-7 Cells. An EcoRI-HindIII fragment of the hCRT-BS2M was subcloned into the expression vector pSV·SPORT1 (GIBCO-BRL). COS-7 cells were transfected with the hCRT-BS2M/pSV·SPORT1 using either the calcium phosphate or DEAE-dextrane transfection method and incubated at 37°C for 72 hours. The cells were then washed once with HEPES-buffered saline (150mM NaCl, 10mM HEPES, 1mM $CaCl_2$, 10mM glucose, 5mM KCl, 1mM $MgCl_2$, (pH 7.4]) and allowed to equilibrate for 10 minutes in the same buffer at 37°C. The medium was then removed and saturation studies were conducted with solutions containing [^{14}C]creatine(5-120 μM, 2.8 mCi/mmol, Amersham) For inhibition studies, [^{14}C]creatine (50 mM) and the appropriate inhibitors were added to the cells in HEPES-buffered saline. Plates were incubated at 37°C for 20 minutes, then washed rapidly three times with HEPES-buffered saline. Cells were solubilized overnight with 1 ml of 10% SDS; 0.5 ml aliquots were removed, and radioactivity was determined by scintillation counting.

RESULTS AND DISCUSSION

To identify novel transporter proteins expressed in mammalian brain, synthetic degenerate primers derived from conserved amino acid sequences flanking TMs II and VI from previously published neurotransmitter transporters (NE, GABA, DA, 5HT, proline and glycine) were synthesized. A single PCR reaction product was obtained from human the human brainstem/spinal

cord cDNA library. The size of the PCR product was 700bp, which is the expected size based on the sequences of the previously published neurotransmitter transporters. The nucleotide sequence of the PCR product had highest homology (97%) with a rat creatine transporter (ratCRT) recently cloned in our laboratory (Fig. 3). Using plaque hybridization techniques, twenty-two clones were isolated from the human brainstem/spinal cord cDNA library. One unique clone, hCRT-BS2M, was sequenced and further characterized.

Fig. 1 shows the nucleotide and predicted amino acid sequence of the human creatine transporter (hCRT-BS2M)..The clone included an open reading frame of 1,905 bp within a 2,283 bp cDNA. The first ATG in the cDNA was assigned as the initiation codon on the basis of the initiation consensus sequence of Kozak (26). The predicted open reading frame encodes a protein of 635 amino acids with a molecular mass of 70,706 Da. Searches performed through Genbank and EMBL databases revealed that the nucleotide sequence was novel. The amino acid sequence analysis revealed that the hCRT-BS2M had the highest homology to the ratCRT (99%) and rbtCRT (97%) and moderate homology to the taurine (53%), GABA/betaine transporters(48-50%), and the glycine, proline, catecholamine and serotonin transporters(40-43%). When compared with a second clone that we isolated from a human striatum cDNA library (hCRT-ST5), the sequence of the hCRT-BS2M from human brainstem showed only two different amino acid residues (Asp^{193} present in the brainstem clone to Ala^{193} in the striatum clone and Thr^{378} in the brainstem clone to Ala^{378} in the striatum clone).

To define the distribution of the mRNA encoding the creatine transporter, we carried out Northern blot analysis of polyA$^+$ RNA isolated from several human tissues (Fig. 2). Under high stringency conditions, a 4.3 kb mRNA species was detected in the human tissues. The strongest hybridization signals were detected in the skeletal muscle, heart and kidney with weaker signals detected in the brain, placenta and lung. There were no signals in the liver or pancreas. The pattern of tissue expression of the hCRT-BS2M mRNAs matched closely that of the rbtCRT (25).

The alignment of amino acid sequences encoded by the ratCRT, the hCRT-BS2M and the rbtCRT is shown in Fig. 3. Hydropathy analysis using the method of Kyte and Doolittle (27) indicates the presence of twelve hydrophobic domains that may represent membrane-spanning segments. Interestingly, the human clones had sixteen amino acid residues that were different from the rabbit clone while only two amino acids were different from the rat clone. The glutamate residues in TMs II and IX were conserved in the rat, rabbit and human creatine transporters(Glu^{109}, Glu^{444}) and are conserved in all members of the sodium dependent plasma membrane transporter family (23). The glycine residue (Gly^{71}) in TM I is conserved in all of the creatine transporters and the amino acid-like subfamily of transporters (taurine, glycine, GABA/betaine, L-proline). However, the glycine residue is substituted by aspartate in dopamine, norepinephrine and serotonin transporters suggesting that the position and identity of this amino acid is a critical determinant for specific classes of transporters.

The rat and human creatine transporters have two consensus sites for N-glycosylation (28) in a large putative extracellular region between TMs III and IV (Asn^{192} and Asn^{197}) that matched the published rabbit creatine transporter (25) (Fig. 3). Another potential site of N-glycosylation (Asn^{548}) is found in the extracellular loop between TMs XI and XII. The presence of multiple (2-

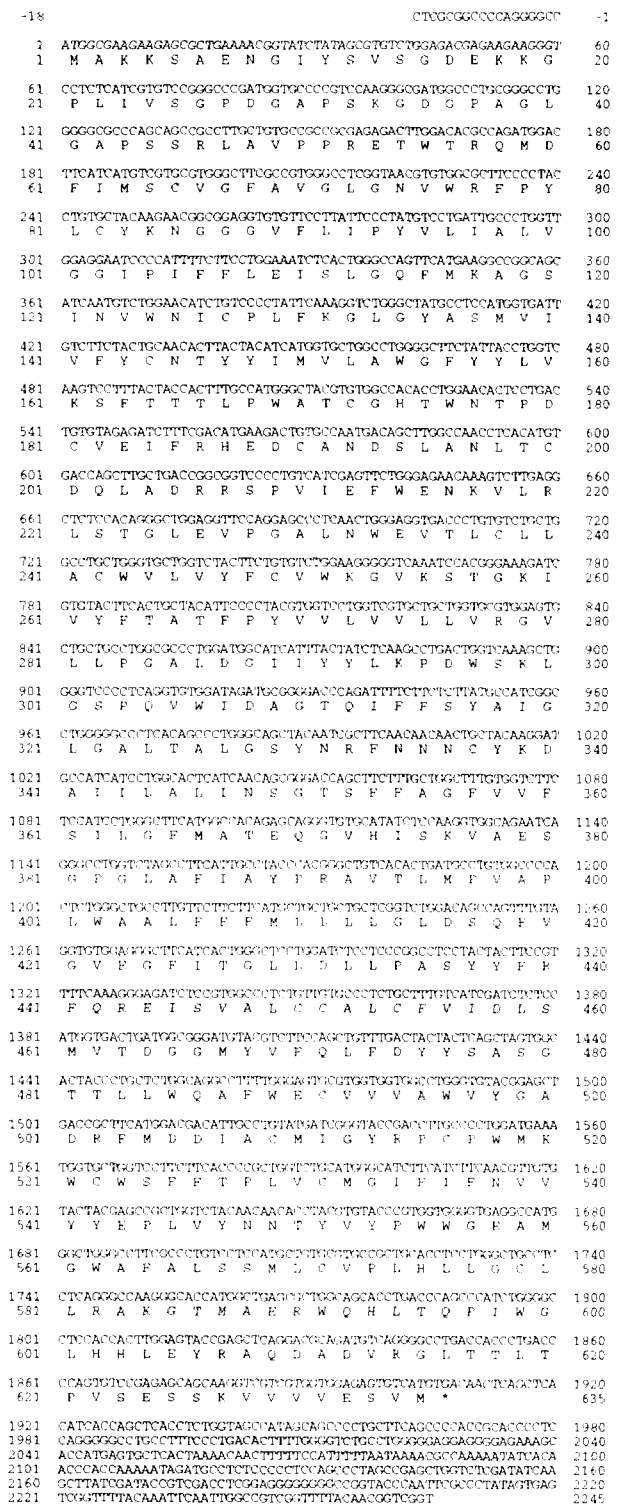

Figure 1. Nucleotide and deduced amino acid sequence of the hCRT-BS2M. The deduced amino acid sequence is shown below the nucleotide sequence. Nucleotides are numbered in the 5' to 3' direction beginning with the first residue of the codon for the putative initiator methionine. The nucleotides on the 5' side of amino acid residue 1 are indicated by negative numbers. The stop codon flanking the open reading frame is denoted with an asterisk.

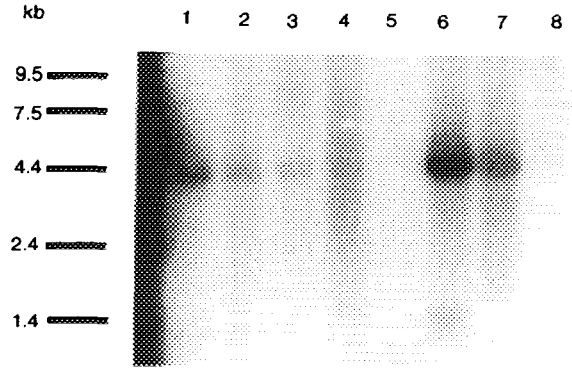

Figure 2. Northern blot analysis of several human tissues. Hybridization was performed as described under "Experimental Procedures." About 2 μg of polyA+ RNA were applied to each well. The size of the transcripts was determined by a RNA standard. The sources of human RNA were as follows: 1) heart, 2)brain, 3)placenta, 4)lung, 5)liver, 6)skeletal muscle, 7)kidney and 8)pancreas.

4) N-linked glycosylation sites in the different transporters also suggests the possibility that variations in glycosylation patterns could contribute to transporter heterogeneity (23). According to the consensus sequencing for phosphorylation sites (29), two putative sites for cAMP-dependent protein kinase (cAMP-PK) were found in the N-terminal region and in the second intracellular loop (Fig. 3). The periodic repeat of leucine residues at every seventh position in the putative third extracellular loop (Leu^{286}, Leu^{293}, Leu^{300} and Ile^{307}) was observed (Fig. 3). This structual feature has been referred to as a "leucine zipper", and was observed in TM II of several glucose transporter glycoproteins, and could be the basis for dimerization of subunits or the site of interaction with other membrane-spanning domains (30). A pattern resembling a leucine zipper is present in TM II of the human noradrenaline (9) and rat serotonin transporters (17), as well as TM IX of the rat (11, 12) and human dopamine transporters (14).

Expression in COS-7 cells demonstrated that cells transfected with the hCRT-BS2M exhibited an average of five-fold increase in [^{14}C]creatine uptake when compared with the non-transfected control ($p<0.05$). The non-transfected control was defined as COS-7 cells exposed to calcium phosphate transfection procedure without DNA. This control had similar levels of [^{14}C]creatine uptake as two different controls which involved transfection of a plasmid without insert, or with the human dopamine transporter cDNA insert (data not shown). Fig. 4 shows [^{14}C]creatine uptake into COS-7 cells transfected with the hCRT-BS2M compared with control uptake in non-transfected cells. Nonlinear regression analysis indicated an average Michaelis constant (K_m) of 14.9±3.0 μM (n=5) for [^{14}C]creatine transport, which is within the range of values in several mammalian tissues and cell types (3-5). The replacement of Na^+ by Li^+ significantly abolished [^{14}C]creatine uptake (95%) (Table 1). The uptake of [^{14}C]creatine significantly inhibited by creatine (91%) and by 3-guanidinopropionate (GP)(Table 1), a well characterized, high affinity alternative substrate for creatine transport in several tissues (31). Other compounds that are able to compete with [^{14}C]creatine for transport were also examined (Table 1). The rank order of potency at a concentration of 5mM was 3-guanidinopropionate (GP) > 4-guanidinobutyrate (GB) > guanidinoacetic acid (GAA) > 2-amino-3-guanidinopropionate (AGP).

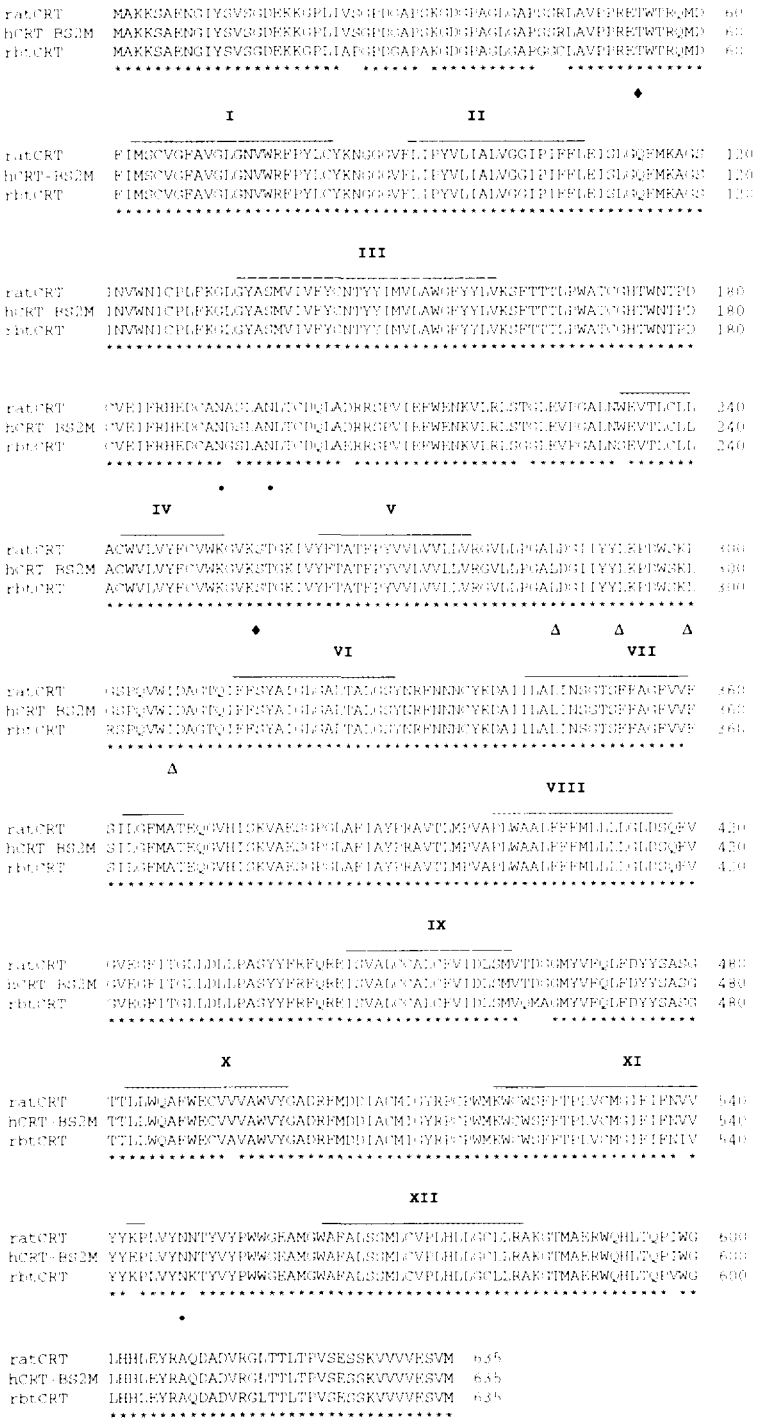

Figure 3. Alignment of the amino acid sequence of the rat (ratCRT), human (hCRT-BS2M) and rabbit (rbtCRT) creatine transporters. The conserved amino acids across all three creatine transporters are indicated by asterisks. Solid lines above the rat creatine transporter sequence reflect the locations of the putative twelve transmembrane domains. The three potential N-linked glycosylation sites are indicated by closed circles (•). The two putative intracellular sites for cAMP-dependent protein kinase (cAMP-PK) phosphorylation are indicated by closed diamonds (♦). The four open triangles (Δ) denote residues defining the conserved leucine zipper motif.

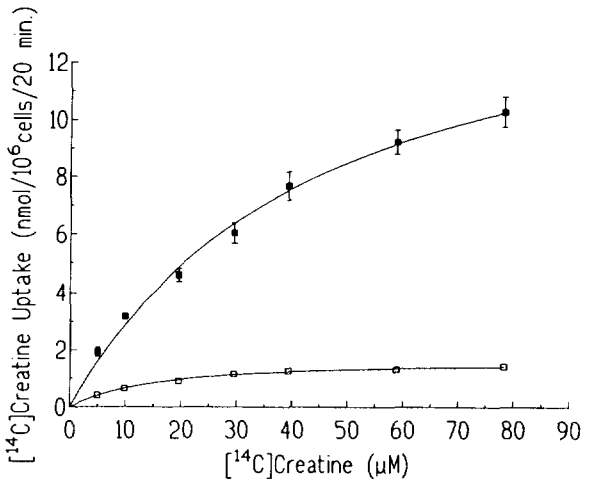

Figure 4. [^{14}C]Creatine uptake into COS-7 cells transfected with hCRT-BS2M (■) or non-transfected cells (□). The assays were carried out with eight different concentrations of [^{14}C]creatine (5 mM to 120 mM). The K_m value for hCRT-BS2M in this experiment was 15.5 mM. The experiment was repeated three times and the average K_m value was 14.9±3.0 mM.

Table 1. [^{14}C]Creatine uptake into transfected COS-7 cells

	[^{14}C]Creatine Uptake	
Treatment	Control	hCRT-BS2M
	(nmol/10^6 cells/20 min.)	
Untreated	0.82 ±0.13*	3.45 ±0.77*†
Li^+	0.04 ±0.01	0.16 ±0.05
Creatine	0.10 ±0.02	0.30 ±0.11
GP	0.06 ±0.01	0.20 ±0.08
GB	0.27 ±0.06	1.07 ±0.32
GAA	0.39 ±0.08	1.42 ±0.37
AGP	0.45 ±0.08	1.68 ±0.43
Choline	0.81 ±0.14	2.96 ±0.59

The uptake was examined using 50 μM [^{14}C]Creatine in the absence (untreated) or presence of inhibitors (5 mM). The inhibitors were creatine, 3-guanidinopropionate (GP), 4-guanidinobutyrate (GB), guanidinoacetic acid (GAA), and 2-amino-3-guanidinopropionate (AGP). The experiment was repeated 5 times in triplicate.

*[^{14}C]Creatine uptake in the corresponding control and hCRT-BS2M transfected cells was statistically different for each of the treatment groups ($p<0.05$).
†[^{14}C]Creatine uptake in treated cells was significantly different from untreated cells within the control and within the hCRT-BS2M transfected group, except for choline treatment ($p<0.05$).

The rank order of potency matches the pharmacological profile shown with the rbtCRT (25). Choline did not significantly inhibit [^{14}C]creatine uptake at a concentration of 5mM ($p>0.05$). Preliminary pharmacological characterization of the second clone from a human striatal cDNA library (hCRT-ST5) revealed the same pharmacological profile as the hCRT-BS2M (data not shown). The significance of the two differing amino acid residues in hCRT-ST5 is not known. Thus, the hCRT-ST5 may represent allelic variation.

Our hCRT-BS2M sequence was 99% identical to the rat creatine transporter clone (CHOT1) as published by Mayser *et al.* (24). We found a significant increase in sodium-dependent [^{14}C]creatine transport. Guimbal *et al.* (25) reported similar results regarding their cloned rabbit creatine transporter. In conclusion, we have cloned a human transporter gene that displays high sequence homology to the ratCRT and rbtCRT (25).

ACKNOWLEDGMENTS

Supported in part by USPHS grants MH-45-51 and program project grant HL-20984. We thank Ms. Pam Abrams for her excellent secretarial support and Dr. Bruno Giros (Duke University) for the dopamine transporter cDNA.

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