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Functional Identification and Molecular Cloning of a Human Brain Vesicle Monoamine Transporter

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Abstract: A vesicle monoamine transporter was functionally identified, molecularly cloned, and characterized from a human substantia nigra cDNA library. The ATP-dependent transport of 5-[^{3}H]hydroxytryptamine ([^{3}H]5-HT) by digitonin-permeabilized fibroblasts expressing the vesicle monoamine/H^+ antiporter in culture exhibited a K_m of 0.55 μM. Reserpine and tetrabenazine, inhibitors of two monoamine binding sites, effectively blocked [^{3}H]5-HT accumulation with K_i values of 34 and 78 nM, respectively. Pretreatment of cells with as little as 10 nM reserpine in the presence of ATP abolished uptake. The rank order for substrate inhibition of [^{3}H]5-HT uptake for both the previously reported rat vMAT1 and the human transporter clone followed the order 5-HT > dopamine > epinephrine > norepinephrine > 1-methyl-4-phenylpyridinium > 2-phenylethylamine > histamine. The virtually identical transport characteristics of rvMAT1 and hvMAT1 confirm the relevance of neuropharmacological studies of rat brain biogenic amine uptake and storage to human brain neurochemistry. **Key Words:** Human vesicle monoamine transporter type I—Vaccinia virus/SP6 bacteriophage hybrid expression—Digitonin permeabilization—Reserpine—Tetrabenazine—Biogenic amines—MPP^+.
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Vesicle transporters allow neurotransmitters to be accumulated from the cytoplasm into storage vesicles, to be made available for regulated exocytotic release. Neurotransmitter accumulation in vesicles depends on transmembrane H^+-electrochemical gradients maintained by an electrogenic vacuolar-type H^+-ATPase (see Njus et al., 1986; Maycox et al., 1990). Because this ATPase is present in all cells, the minimal cellular requirements for reconstitution of vesicle neurotransmitter/H^+ antiport activity can be met within intracellular structures of nonneuroendocrine cells (Erickson et al., 1992), providing a convenient (in vitro) system for cloning, expression, and structural analysis of vesicle transporter cDNAs.

Two vesicle monoamine transporters from rat [here referred to as vesicle monoamine transporter (vMAT1) and chromaffin granule amine transporter (CGAT)/vMAT2] have been obtained by expression cloning techniques using vesicular 5-[^{3}H]hydroxytryptamine ([^{3}H]5-HT) sequestration by intact fibroblasts (Erickson et al., 1992) and resistance to 1-methyl-4-phenylpyridinium (MPP^+) toxicity in epithelioid cells (Liu et al., 1992), respectively. vMAT1 is found in monoamine brainstem nuclei (Erickson et al., 1992; Liu et al., 1992), stomach and basophilic leukemia cells (Erickson et al., 1992), and ganglion cells of the adrenal medulla (Mahata et al., 1993), whereas CGAT/vMAT2 is expressed in chromaffin cells and in PC12 cells (Liu et al., 1992; Mahata et al., 1993).

Here, we describe the cloning and functional characteristics of a human vMAT1. A preliminary report of this work has appeared (Erickson and Eiden, 1993), as have the primary sequences of two additional putative human vMAT1 cDNAs (Surratt et al., 1993; Lesch et al., 1993, GenBank). The present study represents the initial demonstration of the functional properties of human vMAT1, critical to understanding the effects of drugs and neurotoxins on vesicle biogenic amine transport and storage in the human nervous system.

MATERIALS AND METHODS

A variation of the Okayama–Berg cDNA expression vector (pcdSP6/T7) was used to construct a cDNA library (9×10^6 recombinants) from human substantia nigra (M. J. Brownstein, Laboratory of Cell Biology, NIMH). The library was subdivided (3×10^4 clones) and Southern blots of *Bam*HI plasmid restriction digests from overnight cultures were hybridized with a random-primed ^{32}P-labeled rat vMAT1 coding sequence under standard conditions. Of 48 subpools, 12 had homologous cDNA inserts between ~2 and 5 kb.

Identification of subpools with functional cDNA clones and characterization of human vMAT1 were performed using the recombinant SP6 polymerase vaccinia virus expression system (Usdin et al., 1993). For comparison, rat

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Abbreviations used: CGAT, chromaffin granule amine transporter; DA, dopamine; EPI, epinephrine; HIS, histamine; 5-HT, 5-hydroxytryptamine; MPP^+, 1-methyl-4-phenylpyridinium; NE, norepinephrine; PE, 2-phenylethylamine; RES, reserpine; TBZ, tetrabenazine; TM, transmembrane; vMAT, vesicle monoamine transporter.

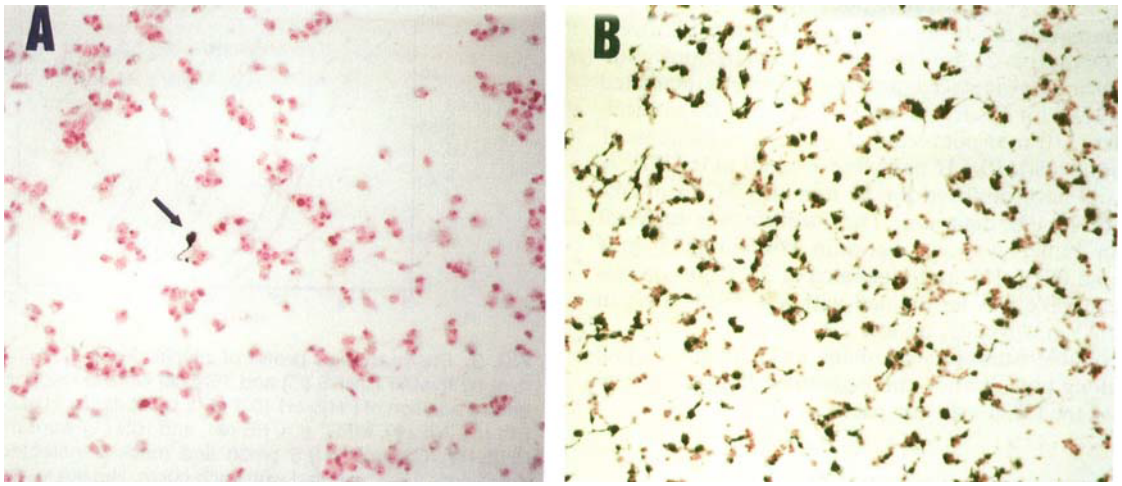

FIG. 1. A: Microscopic identification of functional cDNA for vesicle transport of [^{3}H]5-HT in permeabilized CV-1 cells. Bright-field microscopy shows a positive cell (arrow) observed in one of 12 pools of 30,000 recombinants. **B:** Accumulation of [^{3}H]5-HT (15 min) by CV-1 cells transfected with purified human vMAT1. Slides were processed for autoradiography after fixation of accumulated [^{3}H]5-HT with 2.5% glutaraldehyde and 1:100 acrolein in buffer on ice for 45 min and were under emulsion at 4°C for 5 days. Counterstain was nuclear fast red.

vMAT1 was expressed using the T7 polymerase vaccinia system (Fuerst et al., 1986). Monkey kidney fibroblasts (CV-1 cells) were grown on collagen-coated chamber slides or 35-mm-diameter dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, antibiotics, and glutamine. Cells were infected with virus at 10 plaque-

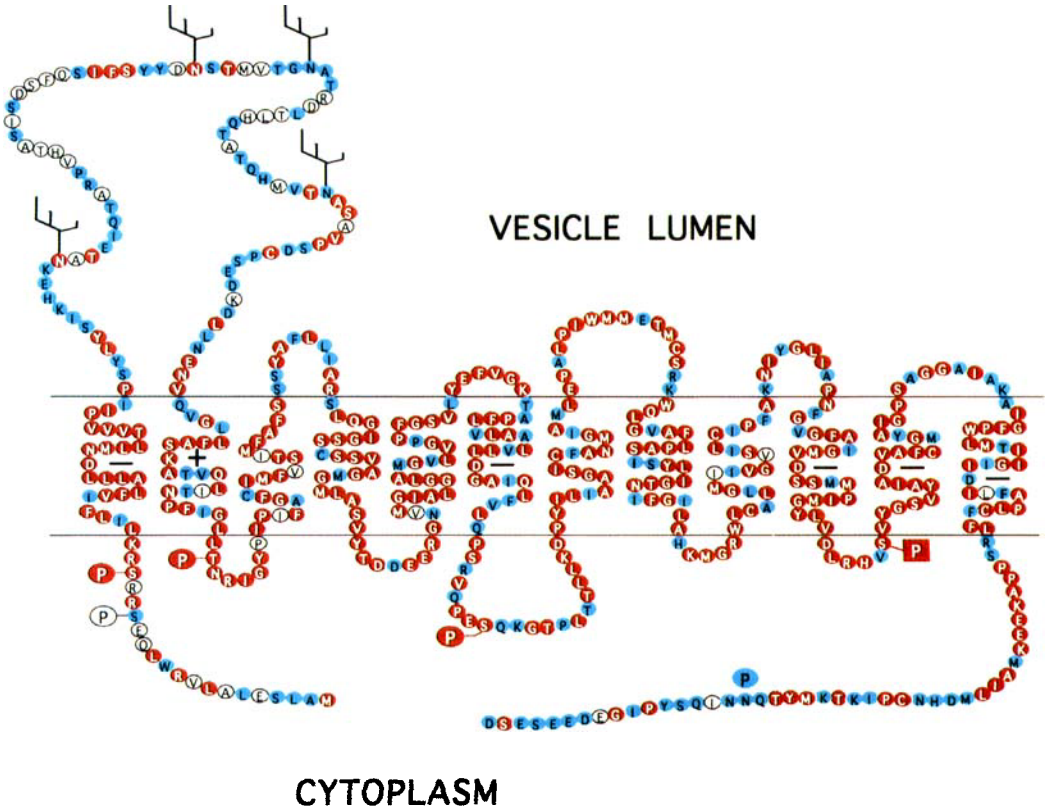

FIG. 2. Predicted amino acid sequence and proposed structure of vMAT1 within the synaptic vesicle membrane. Twelve putative TM domains (I–XII), potential sites for N-linked glycosylation and phosphorylation by protein kinase C (Ⓟ) and protein kinase A (🅿), are indicated. Red indicates amino acids conserved between rat and human vMAT1 and rat CGAT/vMAT2, blue indicates amino acids unique to rat and human vMAT1, and white indicates amino acids unique to human vMAT1. The amino acid sequence differs from that reported by Lesch et al. (1993, Genbank) (X71354*) with Arg^{17} to His and from that reported by Surratt et al. (1993) (L09118*) at the following positions: Cys^{302} to Ser, Lys^{354} to Thr, Ala^{378} to Pro, and Ile^{395} to Asn. The accession number* for human vMAT1 is L23205.

forming units/cell for 30 min, the medium was removed, plasmid cDNA (0.5–2 μg/ml) was transfected by lipofection for 3 h, serum was replaced, and the cells were incubated further at 37°C for 12–16 h. Cells were rinsed with intracellular buffer (110 m*M* potassium tartrate, 5 m*M* glucose, 1 m*M* ascorbic acid, 10 μ*M* pargyline, and 20 m*M* HEPES, pH 7.2) and permeabilized for 10 min at 37°C in uptake buffer with 10 μ*M* digitonin. The medium was replaced with fresh buffer without digitonin containing 5 m*M* MgATP and [^{3}H]5-HT and incubated at 37°C for various intervals. Uptake was terminated with a 1.25-ml wash in buffer with 2 m*M* $MgSO_4$ on ice.

cDNA double-stranded sequencing was performed using primers along both strands and Sequenase II and was analyzed using the GCG software package.

RESULTS AND DISCUSSION

We transfected fibroblasts with 30,000 clone human substantia nigra cDNA sublibraries following infection with the recombinant/SP6 vaccinia expression vector and identified by microscopy clones that conferred ability to accumulate [^{3}H]5-HT following digitonin permeabilization (Fig. 1A). This expression system together with homology screening with rat vMAT1 was used to isolate a ~4.0-kb cDNA vesicle transporter clone.

The predicted amino acid sequence and proposed structure of human vMAT1 within the synaptic vesicle membrane are shown in Fig. 2. Human and rat vMAT1 amino acid sequences are 97% conserved within the putative transmembrane (TM) regions, whereas only 73% identity exists in the glycosylated loop between TM domains I and II. Within the assigned TM domains four conserved aspartic acid residues (I, VI, X, and XI) and one lysine residue (II) are located. Sequence conservation of charged and polar (e.g., Ser) amino acids within the 12 putative TM helices of vMATs suggests their potential involvement in maintaining native conformations of the transporter, in particular for substrate binding and monoamine/H^+ antiport. vMAT amino acids conserved between species but not between vMAT1 and CGAT/vMAT2 may relate to differences in pharmacology of monoamine transport and/or to intracellular addressing of these proteins to synaptic vesicles versus chromaffin granules. Structural homology to putative transport molecules for acetylcholine and xenobiotic molecules within the TM domains indicates that vMATs may be part of a larger family of vesicular neurotransmitter/H^+ antiporters (Liu et al., 1992; Alfonso et al., 1993).

In permeabilized CV-1 cells expressing vMAT1 cDNA >50% of the cells express a functional [^{3}H]5-HT vesicle transport system (Fig. 1B). Transport of [^{3}H]5-HT was linear for 10 min, and maximal levels were stable for >30 min. Uptake was energy dependent because uptake was reduced by >80% in the absence of ATP and abolished by the proton-translocating ionophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone or by inhibition of the vacuolar H^+-ATPase with tri-*n*-butyltin. Specific uptake was not observed with intracellular buffer in the absence of digitonin or in permeabilized CV-1 cells, which express Na^+-dependent plasma membrane transporters. This expression system enables monoamines to be accumulated directly by vMAT1 within an intracellular compartment that contains an electrogenic proton pump (Erickson et al., 1992).

Reserpine (RES) and tetrabenazine (TBZ) are potent inhibitors of [^{3}H]5-HT uptake by vMAT1 (Fig. 3). These drugs are thought to define two conformations of the vesicle monoamine transporter: a charging state (R state), accessible from the cytoplasm, and a discharge state (T state), which may release substrates into the vesicle lumen (Darchen et al., 1989). The conformational shift of vMAT and monoamine/H^+ antiport coupling is dependent on the presence of a H^+ electrochemical gradient across the vesicle membrane. Monoamines bind with high affinity to sites in the R conformation and bind with low affinity to sites in the T conformation, as judged by competition with radiolabeled RES and dihydroTBZ, respectively (Henry et al., 1987). The potency of RES to inhibit [^{3}H]5-HT uptake by vMAT1 is increased ~10-fold following pretreatment of permeabilized cells with ATP. The potency of TBZ to inhibit [^{3}H]5-HT uptake, on the other hand, is unaffected by transmembrane H^+ gradients. TBZ appears to be a more effective inhibitor of human and rat vMAT1 (Erickson et al., 1992) than of rat CGAT/vMAT2 (Liu et al., 1992), perhaps explaining why CNS monoamines are more effectively depleted than peripheral amines by TBZ (Carlsson and Lindqvist, 1966).

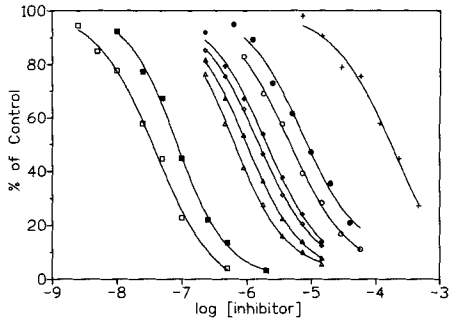

FIG. 3. Pharmacologic profile of inhibition of [^{3}H]5-HT uptake (3 min) by hvMAT1. RES (□) and TBZ (■) were added immediately before addition of [^{3}H]5-HT (0.1 μ*M*). Unlabeled 5-HT (△), DA (▲), EPI (◇), NE (◆), MPP^+ (○), PE (●), and HIS (+) were mixed with [^{3}H]5-HT. Control (~8.5 pmol) and mock transfected (~0.45 pmol) cells were included with each curve. Uptake in mock transfected cells was subtracted from all points. Data are averages of at least three experiments performed in duplicate or quadruplicate. PE and HIS experiments were repeated twice.

Comparison of the potency of various substrates to inhibit [^{3}H]5-HT uptake revealed a rank order of 5-HT > dopamine (DA) > epinephrine (EPI) > norepinephrine (NE) > MPP^+ > 2-phenylethylamine (PE) > histamine (HIS) (Fig. 3). The affinities for the transport site as revealed by inhibition of [^{3}H]5-HT uptake were relatively the same for all substrates tested with human and rat vMAT1 cDNAs (Table 1). The K_i for NE is slightly greater than that for EPI, in contrast to our previous preliminary estimate with NE being more potent than EPI at inhibiting [^{3}H]5-HT transport (Erickson et al., 1992). The rank order of uptake inhibition (5-HT > EPI > DA > NE) and MPP^+ inhibition of [^{3}H]RES binding (IC_{50} = 500 μ*M*) to rat CGAT/vMAT2 has also been reported (Liu et al., 1992; Schuldiner et al., 1993). Whether the apparent differences in these properties of vMAT1 and CGAT/vMAT2 are due to differences in the assay methods used in different laboratories or actual differences between the transporters remains to be determined.

The transport affinity of PE for both the rat and human

TABLE 1. K_i *values of substrates for human and rat CNS vesicle monoamine transporters*

	hvMAT1	rvMAT1
5-HT	0.55 ± 0.05	0.48 ± 0.04
DA	0.82 ± 0.08	0.74 ± 0.03
EPI	1.34 ± 0.23	1.07 ± 0.08
NE	1.82 ± 0.17	1.49 ± 0.19
MPP^+	4.0 ± 0.4	3.8 ± 0.4
PE	5.8	5.0
HIS	143	129

K_i values (μM) were determined by nonlinear regression using Graph Pad software (Graph Pad, San Diego, CA, U.S.A.). Experiments were performed as described in Fig. 3.

vMAT1 molecules is lower than that of all substrates except HIS (Table 1). The anatomical and intraneuronal distribution of PE in the CNS (Paterson et al., 1990) may reflect in part its ability to utilize vMAT only in neurons within which it can compete with other biogenic amines for vesicular uptake and storage.

HIS has relatively low affinity for vMAT1 (Fig. 3). Rat vMAT1 was originally cloned from a rat basophilic leukemia/cognate mast cell storing both HIS and 5-HT, and the low affinity of HIS described here is consistent with its low affinity for the endogenous HIS vesicle carrier (Kanner and Bendahan, 1985; Ludowyke and Lagunoff, 1986). We therefore postulate that vMAT1 may be the vesicle transporter for HIS in mast cells and histaminergic neurons of the CNS.

The cloning of human vMAT1 and its functional characterization demonstrate that this CNS biogenic amine vesicle transporter is highly conserved between species in both structure and function. The similarity of the rat and human CNS vesicle transporters confirms the relevance of the rat CNS in vivo for study of the neuropharmacology of the human brain and the role of vesicle biogenic amine transport and storage in human neuropsychiatric disease.

Note added in proof: The nomenclature for vesicle monoamine transporters was recently discussed at the Philippe Laudat Conference on Molecular Aspects of Neuronal Transporters in Le Bischenberg-Strasbourg, France (26–30 September, 1993). Participants included: R. H. Edwards (U.S.A), J. D. Erickson (U.S.A), J.-P. Henry (France), B. J. Hoffman (U.S.A), and S. Schuldiner (Israel). It was agreed that the transporter originally called CGAT (Liu et al., 1992) will be referred to as VMAT1, and that the transporter originally called SVAT (Liu et al., 1992) or MAT (Erickson et al., 1992) will be referred to as VMAT2. The present study represents the cloning and functional characterization of human VMAT2, using this new nomenclature.

REFERENCES

Alfonso A., Grundahl K., Duerr J. S., Han H.-P., and Rand J. B. (1993) The *Caenorhabditis elegans unc-17* gene: a putative vesicular acetylcholine transporter. *Science* **261,** 617–619.

Carlsson A. and Lindqvist M. (1966) The interference of tetrabenazine, benzquinamide and prenylamine with the action of reserpine. *Acta Pharmacol. Toxicol. (Copenh.)* **24,** 112–120.

Darchen F., Scherman D., and Henry J.-P. (1989) Reserpine binding to chromaffin granules suggests the existence of two conformations of the monoamine transporter. *Biochemistry* **28,** 1692–1697.

Erickson J. D. and Eiden L. E. (1993) Molecular cloning and expression of a human vesicular H^+/monoamine antiporter. (Abstr.) *J. Neurochem.* **61** (Suppl.), S24B.

Erickson J. D., Eiden L. E., and Hoffman B. J. (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. *Proc. Natl. Acad. Sci. USA* **89,** 10993–10997.

Fuerst T. R., Niles E. G., Studier F. W., and Moss B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83,** 8122–8126.

Henry J.-P., Gasnier B., Roisin M. P., Isambert M.-F., and Scherman D. (1987) Molecular pharmacology of the monoamine transporter of the chromaffin granule membrane. *Ann. NY Acad. Sci.* **493,** 194–206.

Kanner B. I. and Bendahan A. (1985) Transport of 5-hydroxytryptamine in membrane vesicles from rat basophilic leukemia cells. *Biochim. Biophys. Acta* **816,** 403–410.

Liu Y., Peter D., Roghani A., Schuldiner S., Prive G. G., Eisenberg D., Brecha N., and Edwards R. H. (1992) A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. *Cell* **70,** 539–551.

Ludowyke R. I. and Lagunoff D. (1986) Amine uptake into intact mast cell granules *in vitro*. *Biochemistry* **25,** 6287–6293.

Mahata S. K., Mahata M., Fischer-Colbrie R., and Winkler H. (1993) Vesicle monoamine transporters 1 and 2: differential distribution and regulation of their mRNAs in chromaffin and ganglion cells of rat adrenal medulla. *Neurosci. Lett.* **156,** 70–72.

Maycox P. R., Hell J. W., and Jahn R. (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. *Trends Neurosci.* **13,** 83–87.

Njus D., Kelley P. M., and Harnadek G. J. (1986) Bioenergetics of secretory vesicles. *Biochim. Biophys. Acta* **853,** 237–265.

Paterson I. A., Juorio A. V., and Boulton A. A. (1990) 2-Phenylethylamine: a modulator of catecholamine transmission in the mammalian central nervous system? *J. Neurochem.* **55,** 1827–1837.

Schuldiner S., Liu Y., and Edwards R. H. (1993) Reserpine binding to a vesicular amine transporter expressed in Chinese hamster ovary fibroblasts. *J. Biol. Chem.* **268,** 29–34.

Surratt C. K., Persico A. M., Yang X.-D., Edgar S. R., Bird G. S., Hawkins A. L., Griffin C. A., Li X., Jabs E. W., and Uhl G. A. (1993) A human synaptic vesicle monoamine transporter cDNA predicts postranslational modifications, reveals chromosome 10 gene localization and identifies *TaqI* RFLPs. *FEBS Lett.* **318,** 325–330.

Usdin T. B., Brownstein M. J., Moss B., and Isaacs S. N. (1993) SP6 RNA polymerase containing vaccinia virus for rapid expression of cloned genes in tissue culture. *Biotechniques* **14,** 223–224.