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## Expression and function of the rat vesicular monoamine transporter 2

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**Adam Y, Edwards RH, Schuldiner S.** Expression and function of the rat vesicular monoamine transporter 2. *Am J Physiol Cell Physiol* 294: C1004–C1011, 2008. First published February 20, 2008; doi:10.1152/ajpcell.00348.2007.—The vesicular monoamine transporters (VMATs) are essential proteins, involved in the storage of monoamines in the central nervous system and in endocrine cells, in a process that involves exchange of 2H<sup>+</sup> with one substrate molecule. The VMATs interact with various native substrates and clinically relevant drugs and display the pharmacological profile of multidrug transporters. Vesicular transporters suffer from a lack of biochemical and structural data due to the difficulties in their expression. In this work we present the high-level expression of rat VMAT2 (rVMAT2) in a stable human embryonic kidney cell line (HEK293), generated using the resistance to the neurotoxin 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) conferred by the protein. In addition, we describe novel procedures for the solubilization and purification of active protein, and its reconstitution into proteoliposomes. The partially purified protein in detergent binds the inhibitor tetrabenazine and, after reconstitution, displays high levels of Δμ<sub>H<sup>+</sup></sub>-driven electrogenic transport of serotonin. The reconstituted purified rVMAT2 has wild-type affinity for serotonin, and its turnover rate is ~0.4 substrate molecule/s.

membrane protein; ion-coupled transporters; neurotransmitter storage; monoamines

CLASSICAL NEUROTRANSMITTERS are stored in synaptic vesicles and the storage granules of secretory cells. Vesicular transporters that use the proton gradient formed by the V-type ATPase mediate this storage process. The vesicular transport of the monoamines—serotonin, dopamine, norepinephrine, epinephrine, and histamine—is mediated by the vesicular monoamine transporter family (VMATs) (24).

Two mammalian genes encode vesicular monoamine transporters that share ~60% homology. Both proteins exchange two protons per substrate molecule, but they display different pharmacological profile and tissue distribution (3, 4, 18). The VMAT2 protein displays higher affinity toward all native substrates and also to the potent specific inhibitor reserpine. VMAT1 is insensitive to the noncompetitive inhibitor tetrabenazine while VMAT2 shows nanomolar affinity. In addition, VMAT1 displays two orders of magnitude lower affinity toward histamine compared with VMAT2 (18). Immunohistochemistry showed that VMAT2 is the more common isoform in most tissues, and it is the only one expressed in neuronal cells, while VMAT1 is found only in some types of endocrine cells. Both genes are expressed in chromaffin cells of the human adrenal medulla (4). VMAT2 is an essential protein, and homozygous VMAT2 knockout mice die shortly after birth (5, 31, 35).

Early biochemical work on monoamine storage focused mainly on chromaffin granules from bovine adrenal medulla. The amine transporter from the chromaffin granules was purified using classical techniques, and two isoforms with different isoelectric points have been characterized (28) that apparently correspond to VMAT1 and VMAT2 (10, 29).

The VMATs are members of the major facilitator superfamily (MFS). This is a ubiquitous and large superfamily of transporters that carry a wide range of substrates and ions (17). High-resolution structures of two proteins from this superfamily are now available (1, 11). The sequence identity between both proteins is only 21%, and their mechanism of action appears to be different. Despite these differences, both structures show a highly similar fold that is, in general, similar to the overall low-resolution structure of OxlT, based on cryo-electron microscopy images (9). The findings suggest the intriguing possibility that the fold of these transporters constitutes a scaffold for all MFS transporters with 12 helices. While the fold is conserved, the specific function is catalyzed by varying sets of amino acids at the substrate binding and translocation domains. This similarity suggests the existence of structural conservation among the superfamily. The VMATs share low sequence homology with the two solved proteins, and on the basis of the structural conservation assumption, a three-dimensional model of the VMAT2 structure was built using the method of comparative modeling (33). The suggested model is in agreement with some existing experimental data about the structure of VMAT and can be the basis for future biochemical research.

In addition to the native substrates, the VMATs interact with many clinically relevant drugs, including the psychostimulants 3,4-methylenedioxymethamphetamine (MDMA) and amphetamines and the parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>). Expression of VMAT confers resistance of mammalian cells to MPP<sup>+</sup>, and this resistance is accomplished by transport and storage in intracellular acidic compartments, thus removing it from its presumed target (14, 15). In addition, some gain of function haplotypes in the human VMAT2 gene were found to protect against Parkinson disease in women, possibly by mitigating the toxicity of dopamine (6). Another clinically relevant drug is the noncompetitive VMAT2 inhibitor tetrabenazine, which is used in the treatment of various movement disorders including Tourette syndrome and Huntington chorea (12). VMAT interacts with various substrates of multidrug transporters, including ethidium, tetraphenylphosphonium, rhodamine, and doxorubicin, suggesting that VMAT has the pharmacological profile of a multidrug transporter (39).

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After the cloning of the two VMAT genes, research focused on protein heterologously expressed in various mammalian cell lines (e.g., Refs. 16, 23, and 25). This research was done in whole cells or vesicles containing many other proteins, and the energy source was provided by the native V-type H<sup>+</sup>-ATPase. In these experiments, many factors could not be controlled. To further understand the mechanism of transport and the interaction with the various distinct drugs, functional purified protein in a reconstituted system is needed for modern biophysical methods, and in the future also for structural research. Native VMAT from bovine adrenal medulla was purified to homogeneity (28, 29). Attempts to work with recombinant purified protein were pursued, with VMAT expressed in the yeast *Saccharomyces cerevisiae* (40) and in insect cells (27). The recombinant protein was purified using a His-tag and showed dihydrotetrabenazine (TBZOH) binding activity when solubilized in detergent, but no transport activity was detectable.

In this work we describe the expression of His-tagged rat VMAT2 in mammalian human embryonic kidney stable cell line (HEK293), generated on the basis of MPP<sup>+</sup> resistance conferred by VMAT2. These fast growing cells express relatively high amounts of protein. Using this expression system, we optimized conditions for the solubilization and immobilization of active protein to nickel-nitrilotriacetic acid beads (Ni-NTA) and identified detergents that preserve VMAT2 activity. In addition, we have developed a protocol for the reconstitution of the partly purified VMAT2 in proteoliposomes. The reconstituted protein displays high levels of electrogenic transport activity and the native affinity to serotonin. Using this system, we determined the turnover rate of the transporter.

## MATERIALS AND METHODS

**Plasmids.** Rat VMAT2 gene (Slc18a2), with hemagglutinin (HA) tag in the second loop between positions 96 and 105, was cloned into pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) with a Myc epitope and six His residues at the COOH terminus. The Myc-His tag was separated from the gene with a cleavage site for the tobacco etch virus (TEV) protease, cloning was done using PCR, with *EcoRI* restriction sites at both sides of the gene and with *BamHI* site after the tags. This construct will be termed "HA-rVMAT2-MH."

**Cell culture.** HEK293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Biological Industries, Beit HaEmek, Israel) and 100 U/ml penicillin-0.1 mg/ml streptomycin, at 37°C under 5% CO<sub>2</sub> atmosphere.

**Generation of stable cell line in HEK293 cells.** VMAT2 protects cells from the toxicity of MPP<sup>+</sup> (14), and this ability was used to generate a cell line based on the protein's function instead of using antibiotic markers. HEK293 cells were tested for the length of their survival in increasing concentrations of MPP<sup>+</sup>, and it was found that in 500 μM MPP<sup>+</sup> the cells died after 7 days. To generate stable cell line, 50% confluent HEK293 cells were transfected with the HA-rVMAT2-MH construct using EscortV (Sigma, St. Louis, MO). At 24 h posttransfection, cells were split into five plates in the ratios of 1:2, 1:5, 1:10, 1:15, and 1:20, and 24 h later, selection with 500 μM MPP<sup>+</sup> started. Medium was changed every 3–4 days for 3 wk. Three resistant colonies were isolated and grown. One colony was used throughout the work and was termed "293-rV2." The cell line was maintained with 500 μM MPP<sup>+</sup> in the standard conditions described above.

For harvesting, 293-rV2 cells were grown in 75-cm<sup>2</sup> T-flasks to 90% confluency, were washed once with 5 ml PBS (37°C) in the plate, resuspended by shaking the flask in 10 ml PBS, and centrifuged 6 min in 800 g at 4°C. The pellet was resuspended in 400 μl ice-cold lysis

buffer containing 320 mM sucrose, 10 mM K-HEPES pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM PMSF, 4 μl protease inhibitors cocktail (Sigma), and 15 μg/ml DNase. Cells were frozen in liquid air and stored in -70°C until used.

For the preparation of cell lysates, frozen cells were thawed, sonicated in ice-cold bath type sonicator for 90 s, and centrifuged 3 min in 1700 g. The supernatant was refrozen in liquid air and kept in -70°C.

**Western blotting.** Samples were mixed with protein sample buffer, and proteins were separated by SDS-PAGE on 12.5% Laemmli gels (13). The protein was transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) by using a semidry blotter for 45 min at 1 mA/cm<sup>2</sup>. Transfer buffer contained 48 mM glycine, 25 mM Tris·HCl (pH 8.3), and 10% methanol. The membrane was then blocked for 1 h in 1% BSA in TBS-T (137 mM NaCl, 50 mM Tris·HCl pH 7.5, and 0.05% Tween 20). The blot was then incubated overnight with 1:5,000 dilution of monoclonal antibody against the HA epitope (12CA5; BAbCo, Berkeley, CA). After five washes with TBS-T, the blot was incubated with anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), developed using SuperSignal HRP substrate (Pierce, Rockford, IL), and imaged using a Fujifilm LAS-1000 imaging system.

**Binding of [<sup>3</sup>H]TBZOH.** Cell lysates (5.4 μl) or liposomes (1 μl) were added to 200 μl of reaction buffer containing 150 mM NaCl, 15 mM Tris·HCl pH 7.5, and increasing concentrations of [<sup>3</sup>H]TBZOH (American Radiolabeled Chemicals, St. Louis, MO) at room temperature. The reaction was stopped after 20 min by dilution in ice-cold buffer without radiolabeled ligand and with 100 μM tetrabenazine and was filtered through 0.22-μm GSWP filters (Millipore) presoaked with 100 μM tetrabenazine. Nonspecific binding measured in the presence of 100 μM tetrabenazine was subtracted from the total binding levels.

**Immunocytochemistry.** Nontransfected HEK293 cells and 293-rV2 cells were grown to 50% confluence on poly-L-lysine-coated coverslips, washed once with PBS, and fixed 40 min with 4% paraformaldehyde. Cells were permeabilized 10 min with 10 μM digitonin in PBS and blocked with 5% skim milk for 1 h. First antibodies monoclonal anti-HA, (12CA5-ABCAM, Cambridge, UK), monoclonal anti-Myc (9E10, Harlan Biotech, Israel), or monoclonal anti-His (BAbCo) were diluted 1:100 in blocking solution and incubated with the cells for 2 h. After three washes with PBS, Cy2-conjugated donkey anti-mouse IgG second antibody (Jackson ImmunoResearch, West Grove, PA) was diluted 1:100 in blocking buffer and incubated with the cells for 1 h. Coverslips were sealed on microscope slides, and cells were scanned using an FV-1000 confocal microscope (Olympus) equipped with an IX81 inverted microscope. A ×40/1.3 oil immersion objective was used. Cy2 fluorescence was imaged using 488-nm laser for excitation and a 505- to 525-nm filter to collect the emission; transmitted light differential interference-contrast images were taken as well.

**Uptake of [<sup>3</sup>H]serotonin in intact cells.** 293-rV2 cells were grown on 25-cm<sup>2</sup> T-flasks, washed with 5 ml PBS, and harvested by shaking in reaction buffer at 37°C. The reaction buffer contained 110 mM Na-K-tartrate, 5 mM glucose, 20 mM K-HEPES pH 7.4, 10 μM pargyline, 0.2% BSA, 1 mM ascorbic acid, 5 mM MgCl<sub>2</sub> and 5 mM ATP. Cells were permeabilized 5 min with 10 μM digitonin, and then [<sup>3</sup>H]serotonin (Perkin Elmer, Boston, MA) was added to 100 nM. Cells were divided into 300-μl aliquots and gently shaken at 37°C. Reaction was stopped after the indicated time by dilution in 2 ml ice-cold buffer, and cells were filtered on GF/C glass filters (Whatman, Kent, UK). The radioactivity on the filters was estimated using liquid scintillation. Values for nonspecific uptake measured in the presence of 5 μM reserpine were subtracted from the total transport.

**Solubilization purification and reconstitution.** For standard preparation, cells from two 75-cm<sup>2</sup> dishes were quickly thawed in 37°C and kept on ice. Dodecyl-maltoside (DDM; Glycon, Luckenwalde, Ger-

many) and polar brain lipids (PBL; Avanti, Alabaster, AL) were added to 2% and 0.5 mg/ml, respectively. After 15 min of shaking at 4°C, cells were sonicated 90 s in an ice-cold bath-type sonicator and then incubated for 1 h at 4°C. Cells were centrifuged 15 min in 21,000 g, and the supernatant was bound to Ni-NTA beads (Qiagen, Hilden, Germany) equilibrated with 150 mM NaCl, 15 mM Tris·HCl pH 7.4 (Na buffer) with 0.08% DDM for 30 min at 4°C. Beads were then loaded on a column, washed once with 10 volumes of Na buffer with 0.08% DDM, 0.5 mg/ml PBL, and 10 mM imidazole, and washed three times with 10 volumes of the same solution but with 1% octyl-glucoside (OG) instead of DDM. HA-rVMAT2-MH was eluted using Na buffer with 1% OG, 0.5 mg/ml PBL, and 300 mM imidazole. A 500  $\mu$ l mixture of 5 mg PBL and 0.5 mg aolectin in Na buffer with 1.2% OG was briefly sonicated in a glass tube, mixed with 500  $\mu$ l of eluted protein, and sonicated to clarity. For the removal of the detergent, the mixture was dialyzed overnight against 300 volumes of the appropriate buffer—190 mM NH<sub>4</sub>Cl or 140 mM (NH<sub>4</sub>)<sub>2</sub>-tartrate with 15 mM Tris-Cl or 10 mM K-HEPES pH 7.4. The dialysis buffer was exchanged with fresh buffer for an additional 2 h, and then the mixture was ultracentrifuged 70 min in 200,000 g. The liposomes pellet was resuspended in 150  $\mu$ l of the above buffer, divided into 25- $\mu$ l aliquots, and kept in -70°C.

**[<sup>3</sup>H]TBZOH binding to detergent solubilized protein.** Cells were thawed and solubilized, and VMAT was immobilized onto Ni-NTA beads as described above. After two washes with 200  $\mu$ l of Na buffer with 10 mM imidazole and the indicated detergent, 200  $\mu$ l of 6 nM [<sup>3</sup>H]TBZOH in the same buffer (without imidazole) were added for 30 min at 4°C. Beads were then spun down, and 150  $\mu$ l were sampled to measure the “unbound” fraction. The rest of the buffer was discarded, and after 10 min in elution buffer with 300 mM imidazole at room temperature, 150  $\mu$ l were sampled to measure the “bound” fraction. Radioactivity was measured using liquid scintillation.

**Uptake of [<sup>3</sup>H]serotonin in proteoliposomes.** Liposomes were thawed and sonicated to clarity in a bath-type sonicator. The uptake assay was performed in reaction buffer containing 140 mM K<sub>2</sub>-tartrate or 190 mM KCl (depending on the inner liposome ammonium salt), 10 mM Tricine, 10 mM Tris, and 5 mM MgCl<sub>2</sub>, at pH 8.5. Liposomes (1  $\mu$ l) were diluted into 200  $\mu$ l of reaction buffer with 50 nM valinomycin and the indicated concentration of the radiolabeled serotonin, usually 100 nM [<sup>3</sup>H]serotonin (Perkin Elmer). Nonspecific accumulation of [<sup>3</sup>H]serotonin was measured in the presence of 5  $\mu$ M reserpine and was subtracted from the total transport. The reaction was stopped at the indicated time by dilution of the mixture in 2 ml ice-cold buffer and was filtered on 0.22- $\mu$ m GSWP filters. Radioactivity in the liposomes was measured using liquid scintillation.

## RESULTS

**Generation of an MPP<sup>+</sup>-resistant cell line that expresses active rVMAT2.** VMAT2 protects cells from the toxicity of the neurotoxin MPP<sup>+</sup> and was first cloned on the basis of this ability (14, 15). In this work we used MPP<sup>+</sup> resistance to generate a stable cell line expressing rVMAT2, by selection based directly on the protein’s activity instead of using antibiotic markers. The cells used are human embryonic kidney cells (HEK293), because they are easy to transfect, grow fast, and can be adapted to grow in suspension (19). The construct used contained three tags, HA in the luminal loop between helices 1 and 2, and Myc and His tags at the COOH terminus separated from the gene by a cleavage site for the TEV protease. After transfection with the HA-rVMAT2-MH construct, the HEK293 cells were grown in the presence of 500  $\mu$ M MPP<sup>+</sup>. The clone that survived the selection and grew well was constantly grown with 500  $\mu$ M MPP<sup>+</sup>.

Immunostaining of the resistant cells showed high expression of protein in this cell line (Fig. 1A). The intracellular pattern of expression was similar with three different antibodies against the HA, Myc, and His epitopes, whereas nontransfected cells showed no stain (data not shown).

To estimate the amounts of functional protein in the cells, we measured binding to the radiolabeled substrate [<sup>3</sup>H]TBZOH, an analog of the high-affinity noncompetitive inhibitor of VMAT2, tetrabenazine (22). The cell lysates display nanomolar affinity TBZOH binding (Fig. 1B), with  $K_d$  of ~17 nM. The maximal number of binding sites ( $B_{max}$ ) value of ~0.12 pmol in 1  $\mu$ l of lysate corresponds to ~2.6  $\mu$ g protein in a 75-cm<sup>2</sup> culture dish, or approximately three million copies per cell.

The MPP<sup>+</sup> resistance of the cell line together with TBZOH binding activity suggests that the protein is functional. To further characterize the activity, we measured transport of serotonin. The assay was an adaptation of the transport assay previously used on transient expression in CV-1 cells and included permeabilization of the plasma membrane with digitonin (25). Resuspended 293-rV2 cells were incubated for 5

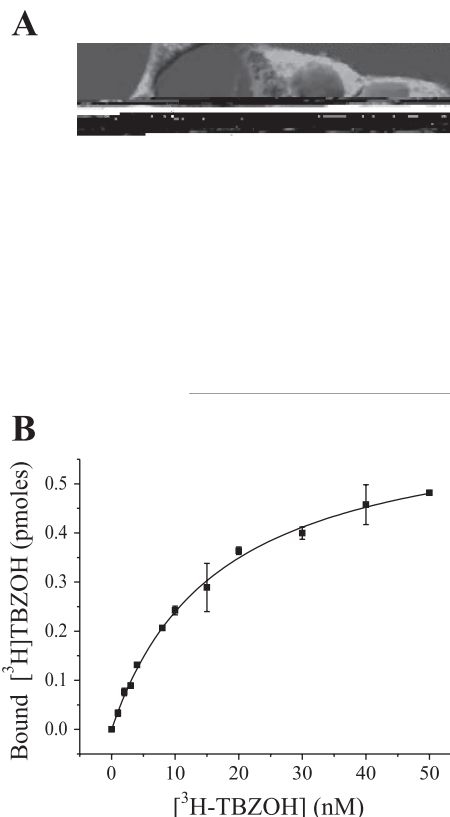


Fig. 1. Expression of functional rat vesicular monoamine transporter 2 (rVMAT2) in 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)-resistant HEK293 cell line (293-rV2). **A:** immunocytochemistry of the 293-rV2 cell line with anti-Myc antibody. Massive stain is visible in intracellular distribution. Similar stain was obtained with anti-hemagglutinin (HA) and anti-His antibodies, whereas nontransfected HEK293 cells showed no fluorescence (data not shown). Scale bar is 10  $\mu$ M. **B:** binding of the noncompetitive inhibitor of VMAT2, [<sup>3</sup>H]dihydrotetrabenazine ([<sup>3</sup>H]TBZOH), to lysates from the 293-rV2 cell line. Constant amount of cell lysates was bound to increasing [<sup>3</sup>H]TBZOH concentrations for 20 min, followed by fast dilution and filtration on 0.22- $\mu$ m filters. Data were fitted to  $K_d$  equation [ $y = B_{max} \cdot x / (K_d + x)$ ], where  $B_{max}$  is maximal number of binding sites. The calculated  $K_d$  is  $16.8 \pm 1.02$  nM, and the  $B_{max}$  is  $0.64 \pm 0.01$  pmol ( $R^2 = 0.997$ ). The presented data are from two experiments, and each point is the average of duplicates.

min with digitonin and ATP and were then assayed for transport of serotonin. As shown in Fig. 2, the cells display high levels of ATP-dependent serotonin transport, whereas non-transfected HEK293 cells display no activity. The serotonin transport is sensitive to reserpine, a specific competitive inhibitor of VMAT, and also to tetrabenazine (data not shown). These results show that the construct used is functional, interacts with various known substrates of VMAT2, and displays high levels of transport activity.

**Solubilization and partial purification of rVMAT2.** To optimize conditions for solubilization and purification of VMAT2, we screened a series of detergents. Initial results suggested that TBZ binding by protein solubilized in DDM is high and to levels similar to those observed in membrane preparation. In the screen for other detergents, we first tested their ability to solubilize VMAT2 and allow activity after replacement with DDM. In these experiments, membrane preparations were solubilized with eight different detergents, VMAT2 was immobilized on Ni-NTA beads, and the detergent was exchanged by washing with DDM (Fig. 3A). Two detergents, Fos-choline 14 and Anzergent 3-14, completely abolish the binding activity after exchange to DDM, whereas with three other detergents, Na-cholate, lauryl dimethylamine oxide (LDAO), and CHAPS, ~50% of binding activity is retained. Two other detergents, OG and Triton X-100, allow binding activity similar to that of the protein originally solubilized in DDM.

To test whether activity can be detected without exchange to DDM, we performed the binding assay in OG and Triton X-100 (Fig. 3B). In Triton X-100, no binding activity could be measured, and in OG only 20% binding activity was detected compared with DDM. The results suggest that even though the detergents do not denature the protein, they do not allow binding activity, so among the detergents tested, DDM is the best to assess activity. As shown in Fig. 3A, solubilization in Fos-choline 14 completely abolished the protein's activity. This effect could be due to the fact that Fos-choline is a very strong detergent that removes essential lipids. We tested whether the activity could be recovered after exchange of the

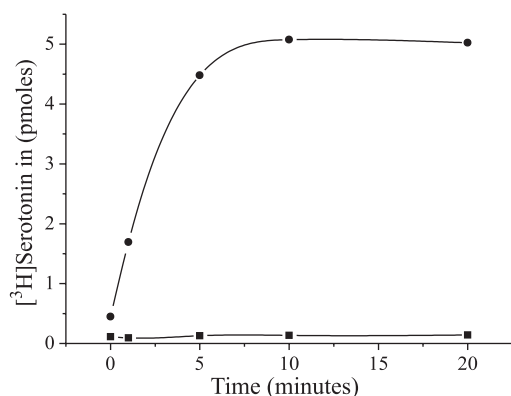


Fig. 2. Uptake of [ $^3\text{H}$ ]serotonin in intact cells. 293-rV2 cells were resuspended and incubated with 10  $\mu\text{M}$  digitonin and 5 mM ATP for 5 min, followed by addition of 100 nM [ $^3\text{H}$ ]serotonin. The reaction was stopped at the indicated time by dilution in cold buffer and filtration on glass filters. Uptake in 293-rV2 cells ( $\bullet$ ) is shown. The presented data are after subtraction of the low nonspecific accumulation of serotonin measured by inhibition with 5  $\mu\text{M}$  of the specific inhibitor reserpine, which is similar to the accumulation levels of the nontransfected HEK293 cells ( $\blacksquare$ ). A typical experiment is presented (this assay was repeated five times).

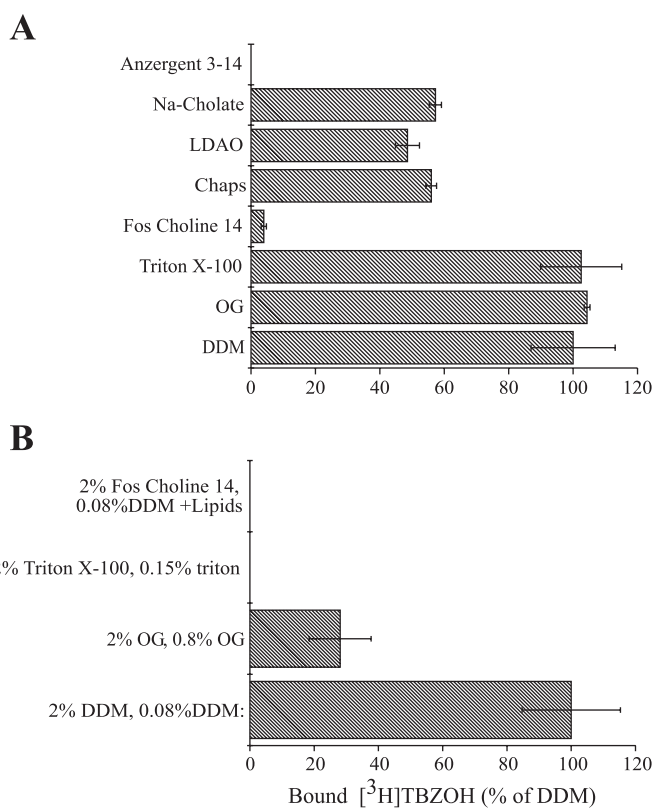


Fig. 3. Solubilization screen for active rVMAT2. **A**: 293-rV2 cells were solubilized with 2% of the indicated detergent for 1 h, followed by 30 min binding to nickel-nitrilotriacetic acid (Ni-NTA) beads. The immobilized protein was washed twice with 10 volumes of 0.08% DDM, and binding for 30 min at 6 nM [ $^3\text{H}$ ]TBZOH was done, followed by elution in 300 mM imidazole. The assay was done in duplicate. **B**: similar experiment, but this time the cells in each bar were solubilized in the first indicated detergent, and then washes and binding were done in the second one at the indicated concentration. This assay was done in triplicate. OG, octyl-glucoside; LDAO, lauryl dimethylamine oxide; DDM, dodecyl-maltoside.

detergent to DDM and addition of brain lipids. The results shown in Fig. 3B demonstrate that the activity is not recovered and suggest that this detergent irreversibly denatures the protein or that it is very difficult to exchange since no recovery is observed.

After choosing DDM as the detergent for solubilization and for activity measurements, we used the TBZOH binding assay as the criterion to screen for the best conditions for immobilization to Ni-NTA, for maximal yield of active protein. The screen included the solubilization time, the amounts of protease inhibitors and metal chelators, and the imidazole concentrations for washes and elution (data not shown). The final conditions are described in MATERIALS AND METHODS.

Figure 4 summarizes the partial purification of VMAT2 from 293-rV2 cells. Coomassie stain of the total lysate and the DDM-soluble fraction shows a complex picture with many proteins, as expected from such a preparation. After immobilization, only a very minor portion of the total cell proteins is visible, even though the fraction loaded is equivalent to four times the amount shown in the solubilize lane. The recombinant protein contains an HA tag, and Western blot analysis of this preparation shows two bands in the  $M_r$  of ~65 kDa and 90 kDa. Two faint bands in similar  $M_r$  are visible also in the

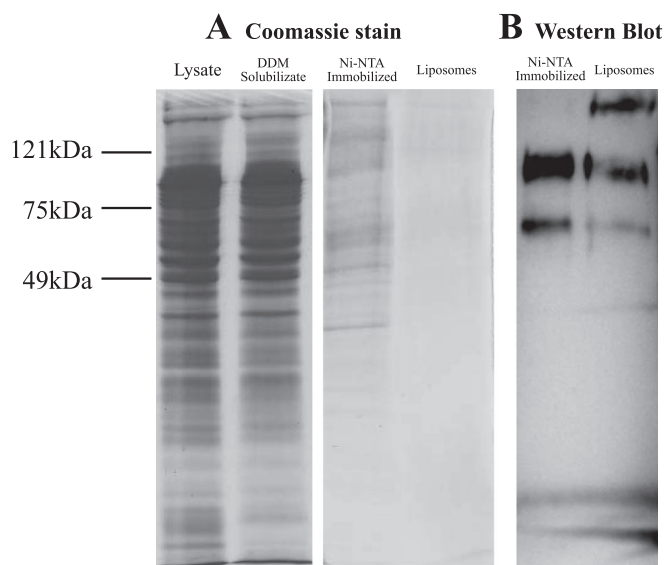


Fig. 4. Partial purification of rVMAT2. *A*: Coomassie stain of the various steps of purification. The protein in the lanes marked "Ni-NTA immobilized" and "liposomes" was obtained from an amount of cells 4 times higher than that used for the lanes of lysates and solubilized cells presented. *B*: Western blot with anti-HA antibody of the same amounts of Ni-NTA immobilized and reconstituted proteins as presented in the Coomassie stain.

Coomassie stain and may correspond to the purified VMAT2. We previously showed for native bovine VMAT2 (28) and recombinant VMAT1 (41) a similar pattern in which glycosylation to different levels results in several species that can be distinguished in Coomassie stain or with antibodies. The non-glycosylated form, with an apparent  $M_r$  of  $\sim 55$  kDa, is not observed in the purified fraction. In the lysate and solubilize, the response to anti-HA is very weak (data not shown), maybe because of the small amounts of VMAT2 in the lysate and the presence of other major proteins in the same area of the gel that disturb the electrotransfer of the protein. No immunoreactivity is visible in lysates from nontransfected HEK293 cells (data not shown). The degree of purification cannot be determined because of the low amounts of protein and the resulting ambiguity in the identification of the protein in Coomassie; however, it is also evident that the fraction is highly enriched with rVMAT2 relative to the initial solubilize, and it was further characterized.

The purified rVMAT2 can be reconstituted into proteoliposomes, and it displays electrogenic transport activity. Binding of tetrabenazine is a good estimate for the activity of the protein, but it does not necessarily indicate a fully active protein capable of transport activity. An example for VMAT that binds tetrabenazine but does not display transport activity is the bovine VMAT2 expressed in yeast (40). Native VMAT purified using classic techniques from bovine chromaffin granules was reconstituted and showed transport activity (28), and in this work we have adapted those protocols to modern detergents and metal-chelate chromatography. A screen of the conditions for reconstitution was done, and the protocol developed is presented in MATERIALS AND METHODS. This protocol is based on the exchange of DDM to the high-critical micellar concentration (CMC) detergent OG that does not denature the protein (Fig. 3), instead of solubilization in Triton and exchange to Na-Cholate as previously done (28). The whole

process was done in the presence of 0.5 mg/ml polar brain lipids, and the protein was reconstituted in a mix of polar brain lipid extract and asolectin. The detergent was removed by overnight dialysis. The proton gradient (acidic inside) was generated by an ammonium gradient (high inside), a procedure that takes advantage of the high permeability of the nonprotonated species that rapidly leaks downhill. Since its concentration in the proteoliposome decreases, the relatively impermeant ammonium dissociates and supplies the protons to be used by the transporter. We used ammonium tartrate salt to get a stable gradient for longer uptake time. Ammonium chloride holds the gradient for no longer than 5 min, most likely because of the relatively higher permeability of the liposomes to chloride ions (data not shown).

The reconstituted protein accumulates serotonin in a time-dependent fashion (Fig. 5A), and this transport is inhibited by reserpine and tetrabenazine (data not shown). In addition, no transport was observed when the proton gradient was abolished by the protonophore nigericin.

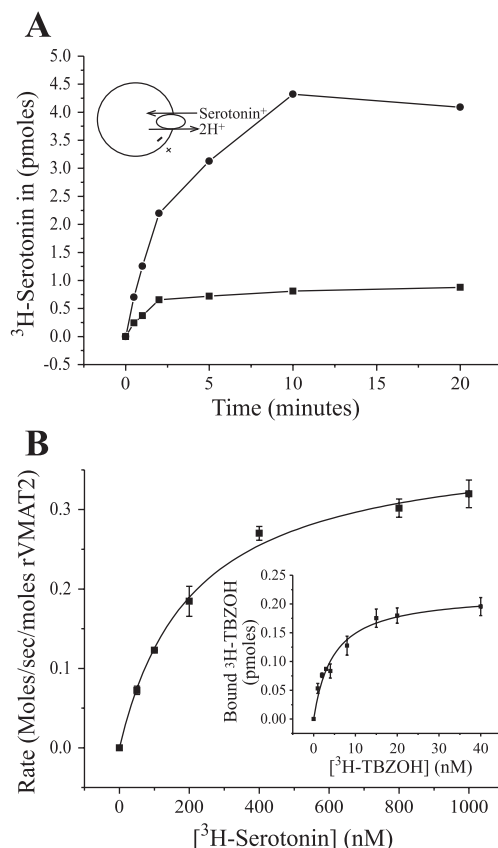


Fig. 5. Uptake of [ $^3$ H]serotonin in rVMAT2 reconstituted in proteoliposomes. *A*: time course of the uptake reaction. Liposomes ( $1 \mu\text{l}$ ) were diluted in reaction buffer at pH 8.5 with 100 nM [ $^3$ H]serotonin with ( $\bullet$ ) and without ( $\blacksquare$ ) 50 nM valinomycin. The results suggest that the transport is electrogenic. A typical experiment is presented, and this assay was repeated 5 times. *B*: Michaelis-Menten fit of concentration-dependent uptake of serotonin. *Inset* shows a binding isotherm of [ $^3$ H]TBZOH to the proteoliposomes. The calculated  $K_d$  is  $4.7 \pm 0.78$  nM, and  $B_{\text{max}}$  is  $0.22 \pm 0.01$  pmol/ $1 \mu\text{l}$  liposomes ( $R^2 = 0.976$ ). We used the  $B_{\text{max}}$  value to calculate the turnover rate from the transport  $V_{\text{max}}$ . The calculated  $K_m$  for serotonin is  $210 \pm 18$  nM, and the turnover rate is  $0.39 \pm 0.01$  mol substrate  $\cdot$  s $^{-1}$   $\cdot$  mol rVMAT2 $^{-1}$ . The  $K_m$  experiment was performed in duplicate and was repeated twice. In the *inset* we present data from two separate experiments; each point was performed in duplicate.

In the absence of valinomycin (Fig. 5A; squares), the background of nonspecific serotonin accumulation (as measured by inhibition with reserpine or tetrabenazine) was very high, at levels of almost 50% of the uptake (data not shown). The background is observed in liposomes without VMAT and is due to unmediated accumulation of the weak base serotonin down the proton chemical gradient. The vesicular monoamine transporter exchanges two protons with one positively charged substrate molecule (24). This electrogenic transport mechanism is expected to produce negative membrane potential inside the liposome that lowers the  $\Delta\mu_{H^+}$  available for transport activity. In the native vacuolar system, this potential is most likely dissipated by other transport systems. When the protein is reconstituted in proteoliposomes, such a negative membrane potential is expected only if the liposomes are tight. To test whether the membrane potential is the reason for the low transport relative to background accumulation, we added valinomycin, an ionophore that increases the permeability of potassium ions and generates a diffusion membrane potential (positive inside). As shown in Fig. 5A (circles), valinomycin induces a dramatic increase in the transport levels, while the background levels in the presence of reserpine were not affected (data not shown). Therefore background serotonin accumulation in these conditions is  $\sim 10\%$  of the total serotonin transport. The results presented imply that the proteoliposomes display low nonspecific permeability to ions and that the protein functions in the expected electrogenic manner.

*The partially purified reconstituted protein displays native affinity and exchanges 0.4 molecules/s.* The affinity of the reconstituted recombinant protein was measured by 1-min transport assays at increasing concentrations of [ $^3H$ ]serotonin (Fig. 5B). From the fit to the Michaelis-Menten equation (Fig. 5B), a  $K_m$  value of  $210 \pm 18$  nM was calculated. This value is similar to the  $K_m$  measured in vesicles isolated from Chinese hamster ovary (CHO) cells stably expressing VMAT2 to  $\sim 190$  nM (18), and  $\sim 10$  times lower than the value measured in native VMAT2 purified from bovine chromaffin granules and reconstituted in proteoliposomes (28).

To further characterize the properties of the protein, we measured the  $K_d$  and  $B_{max}$  for binding of the reconstituted rVMAT2 to [ $^3H$ ]TBZOH. Interestingly, in liposomes the affinity to TBZOH ( $4.7 \pm 0.78$  nM) is slightly higher than in lysates ( $17 \pm 1.02$  nM) and is very close to the affinity measured in bovine chromaffin granules [ $\sim 3$  nM, (22)]. The number of TBZ binding sites in  $1 \mu\text{l}$  proteoliposomes is  $0.22 \pm 0.01$  pmol (Fig. 5B, inset). This number allows us to estimate the turnover rate of the reconstituted protein from the  $V_{max}$  value of the Michaelis-Menten fit (Fig. 4B). The calculated rate suggests that, every second, the purified protein performs  $0.39 \pm 0.01$  transport cycles, or  $\sim 23$  cycles/min. This value is approximately threefold higher than the rate measured for the purified bovine VMAT in the absence of valinomycin (28) and is very similar to that observed for nonpurified heterologous expression in COS cells (18).

## DISCUSSION

The work presented here describes the expression, partial purification, and characterization of rVMAT2. This protein plays a central role in neurotransmission in the brain and the endocrine system and serves as target for many drugs. Native

vesicular neurotransmitters transporters have been extensively characterized at the biochemical level (24). The lack of more detailed high-resolution information is probably due to three main reasons: 1) Like many other mammalian membrane proteins, it is difficult to express high levels of an active protein; therefore no structural data are available. 2) In contrast with plasma membrane neurotransmitter transporters, in which high-quality structures of bacterial homologues of the two main families are available (38, 42), there are no such structures of close bacterial homologues for any vesicular transporter. A homology model of VMAT was proposed based on medium-resolution structures of MFS proteins that display a weak homology to VMAT (33). The technology presented here provides tools to test this model experimentally. 3) Plasma membrane mammalian transporters can be characterized by electrophysiology; this method is very powerful and does not require high levels of expression. However, this method does not seem to be easily applicable to transporters that are expressed in intracellular membranes (some attempts have been made; see Refs. 34 and 37 for further discussion).

For the above reasons, it is necessary to find new expression methods and to develop functional assays for VMAT. Mammalian cell expression systems, although expensive and time consuming, allow for the expression of active protein with all the posttranslational modifications. For example, a screen of expression systems for the plasma membrane serotonin transporter (SERT) showed that in bacteria and yeast, no active transporter is expressed, and in insect cells, the vast majority of expressed SERT was unglycosylated and nonfunctional. In contrast, in four different mammalian expression systems, the protein is fully active (32). As mentioned above, VMAT expressed in yeast and in insect cells displayed only inhibitor binding and not full transport activity (27, 40). After submission of this article, new work was published by the Ruoho group (7, 26) showing that VMAT2 in vesicles isolated from insect SF9 cells display some degree of serotonin transport, suggesting that this system may also be useful for the expression of functional VMAT.

The cell line developed in this work was generated on the basis of the criteria of the proteins' functionality, by selection on the basis of MPP $^+$  resistance conferred by VMAT2. The cells grow well at high concentration of MPP $^+$ , express relatively high amount of active protein, and display uptake of serotonin in intact cells. For comparison, in the best mammalian expression system for SERT, the approximation is 400,000 copies per cell (32), whereas in our cell line, we predict about three million copies of tetrabenazine binding VMAT2 molecules. Stable and transient expression in HEK293 cells was used for the expression of high amounts of various mammalian proteins, and another advantage of these cells is that they can be adapted to grow in suspension, for growth in bioreactors (2, 19, 36).

Besides the expression challenge, membrane proteins create another difficulty for biochemists and structural biologists, because of the need to find the right detergent to keep the protein correctly folded in solution. The vesicular monoamine transporter has been the subject of intense research for many years. In the past, biochemical studies were done mainly on the native protein in bovine chromaffin granules from the adrenal medulla (8, 20–22, 28–30). After cloning of the two VMAT genes, research was performed with protein heterologously

expressed in various mammalian cell lines (e.g., Refs. 16, 23, and 25). This research was done in whole cells or in vesicles containing many other proteins, and the energy source was the native V-type ATPase. Under these conditions, many factors cannot be controlled. In this work we present a screen for solubilization conditions of VMAT2 aimed at keeping it active in solution, by the criterion of tetrabenazine binding. The protocol presented here can be useful for future biochemical and structural study of VMAT with any expression system. In addition, the reconstitution protocol presented here shows that the purified VMAT2 solubilized in DDM does not lose its transport ability and displays the wild-type  $K_m$  to serotonin.

Because of the low amounts of protein after purification in this scale, we cannot determine the degree of purity of VMAT. For structural studies after upscaling of the system or for studies where high purity is desired, an additional purification step will be necessary. The framework for further research was provided here.

The reconstitution of partially purified rVMAT2 allowed us to measure the approximate turnover rate of the transporter. On the basis of the number of TBZ binding sites and the initial rates of transport, we estimate a turnover of  $\sim 0.39$  transport cycles/s. This number is slightly higher than previously determined for the protein purified from adrenal medulla (28).

To conclude, in this work we present methods for the study of the vesicular monoamine transporter. These methods provide tools for testing homology models of VMAT and to understand the mechanism of substrate recognition, coupling, and translocation. These protocols will be also useful in future structural studies of this protein. In addition, we present here the approximate turnover rate of this transporter based on measurements done with partially purified reconstituted protein with wild-type  $K_m$  to serotonin.

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#### REFERENCES

1. Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S. Structure and mechanism of the lactose permease of *Escherichia coli*. *Science* 301: 610–615, 2003.
2. Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* 30: E9, 2002.
3. Eiden LE. The vesicular neurotransmitter transporters: current perspectives and future prospects. *FASEB J* 14: 2396–2400, 2000.
4. Erickson JD, Schafer MK, Bonner TI, Eiden LE, Weihe E. Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. *Proc Natl Acad Sci USA* 93: 5166–5171, 1996.
5. Fon EA, Pothos EN, Sun BC, Killeen N, Sulzer D, Edwards RH. Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. *Neuron* 19: 1271–1283, 1997.
6. Glatt CE, Wahner AD, White DJ, Ruiz-Linares A, Ritz B. Gain-of-function haplotypes in the vesicular monoamine transporter promoter are protective for Parkinson disease in women. *Hum Mol Genet* 15: 299–305, 2006.
7. Gopalakrishnan A, Sievert M, Ruoho AE. Identification of the substrate binding region of vesicular monoamine transporter-2 (VMAT-2) using iodoaminoflissopolol as a novel photoprobe. *Mol Pharmacol* 72: 1567–1575, 2007.
8. Henry JP, Scherman D. Radioligands of the vesicular monoamine transporter and their use as markers of monoamine storage vesicles. *Biochem Pharmacol* 38: 2395–2404, 1989.
9. Hirai T, Heymann JAW, Maloney PC, Subramaniam S. Structural model for 12-helix transporters belonging to the major facilitator superfamily. *J Bacteriol* 185: 1712–1718, 2003.
10. Howell M, Shirvan A, Stern-Bach Y, Steiner-Mordoch S, Strasser JE, Dean GE, Schuldiner S. Cloning and functional expression of a tetrabenazine sensitive vesicular monoamine transporter from bovine chromaffin granules. *FEBS Lett* 338: 16–22, 1994.
11. Huang Y, Lemieux MJ, Song J, Auer M, Wang DN. Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science* 301: 616–620, 2003.
12. Kenney C, Jankovic J. Tetrabenazine in the treatment of hyperkinetic movement disorders. *Expert Rev Neurother* 6: 7–17, 2006.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1970.
14. Liu Y, Peter D, Roghani A, Schuldiner S, Prive GG, Eisenberg D, Brecha N, Edwards RH. A cDNA that suppresses MPP<sup>+</sup> toxicity encodes a vesicular amine transporter. *Cell* 70: 539–551, 1992.
15. Liu Y, Roghani A, Edwards R. Gene transfer of a reserpine-sensitive mechanism of resistance to N-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* 89: 9074–9078, 1992.
16. Merickel A, Kaback HR, Edwards RH. Charged residues in transmembrane domains II and XI of a vesicular monoamine transporter form a charge pair that promotes high affinity substrate recognition. *J Biol Chem* 272: 5403–5408, 1997.
17. Pao SS, Paulsen IT, Saier, MH Jr. Major facilitator superfamily. *Microbiol Mol Biol Rev* 62: 1–34, 1998.
18. Peter D, Jimenez J, Liu Y, Kim J, Edwards RH. The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. *J Biol Chem* 269: 7231–7237, 1994.
19. Reeves PJ, Thurmond RL, Khorana HG. Structure and function in rhodopsin: high level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proc Natl Acad Sci USA* 93: 11487–11492, 1996.
20. Rudnick G, Steiner-Mordoch SS, Fishkes H, Stern-Bach Y, Schuldiner S. Energetics of reserpine binding and occlusion by the chromaffin granule biogenic amine transporter. *Biochemistry* 29: 603–608, 1990.
21. Scherman D. Dihydro-tetrabenazine binding and monoamine uptake in mouse brain regions. *J Neurochem* 47: 331–339, 1986.
22. Scherman D, Jaudon P, Henry JP. Characterization of the monoamine carrier of chromaffin granule membrane by binding of [2-3H]dihydro-tetrabenazine. *Proc Natl Acad Sci USA* 80: 584–588, 1983.
23. Schuldiner S, Liu Y, Edwards RH. Reserpine binding to a vesicular amine transporter expressed in Chinese hamster ovary fibroblasts. *J Biol Chem* 268: 29–34, 1993.
24. Schuldiner S, Shirvan A, Linial M. Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol Rev* 75: 369–392, 1995.
25. Shirvan A, Laskar O, Steiner-Mordoch S, Schuldiner S. Histidine-419 plays a role in energy coupling in the vesicular monoamine transporter from rat. *FEBS Lett* 356: 145–150, 1994.
26. Sievert MK, Hajjipour AR, Ruoho AE. Specific derivatization of the vesicle monoamine transporter with novel carrier-free radioiodinated reserpine and tetrabenazine photoaffinity labels. *Anal Biochem* 367: 68–78, 2007.
27. Sievert MK, Thiriot DS, Edwards RH, Ruoho AE. High-efficiency expression and characterization of the synaptic-vesicle monoamine transporter from baculovirus-infected insect cells. *Biochem J* 330: 959–966, 1998.



28. Stern-Bach Y, Greenberg-Ofrath N, Flechner I, Schuldiner S. Identification and purification of a functional amine transporter from bovine chromaffin granules. *J Biol Chem* 265: 3961–3966, 1990.
29. Stern-Bach Y, Keen JN, Bejerano M, Steiner-Mordoch S, Wallach M, Findlay JB, Schuldiner S. Homology of a vesicular amine transporter to a gene conferring resistance to 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* 89: 9730–9733, 1992.
30. Suchi R, Stern-Bach Y, Gabay T, Schuldiner S. Covalent modification of the amine transporter with *N,N'*-dicyclohexylcarbodiimide. *Biochemistry* 30: 6490–6494, 1991.
31. Takahashi N, Miner LL, Sora I, Ujike H, Revay RS, Kostic V, Jackson-Lewis V, Przedborski S, Uhl GR. VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci USA* 94: 9938–9943, 1997.
32. Tate CG, Haase J, Baker C, Boorsma M, Magnani F, Vallis Y, Williams DC. Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter. *Biochim Biophys Acta* 1610: 141–153, 2003.
33. Vardy E, Arkin IT, Gottschalk KE, Kaback HR, Schuldiner S. Structural conservation in the major facilitator superfamily as revealed by comparative modeling. *Protein Sci* 13: 1832–1840, 2004.
34. Volz TJ, Hanson GR, Fleckenstein AE. Measurement of kinetically resolved vesicular dopamine uptake and efflux using rotating disk electrode voltammetry. *J Neurosci Methods* 155: 109–115, 2006.
35. Wang YM, Gainetdinov RR, Fumagalli F, Xu F, Jones SR, Bock CB, Miller GW, Wightman RM, Caron MG. Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron* 19: 1285–1296, 1997.
36. West M, Park D, Dodd JR, Kistler J, Christie DL. Purification and characterization of the creatine transporter expressed at high levels in HEK293 cells. *Protein Expr Purif* 41: 393–401, 2005.
37. Whitley J, Parsons J, Freeman J, Liu Y, Edwards RH, Near JA. Electrochemical monitoring of transport by a vesicular monoamine transporter expressed in *Xenopus* oocytes. *J Neurosci Methods* 133: 191–199, 2004.
38. Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporters. *Nature* 437: 215–223, 2005.
39. Yelin R, Schuldiner S. The pharmacological profile of the vesicular monoamine transporter resembles that of multidrug transporters. *FEBS Lett* 377: 201–207, 1995.
40. Yelin R, Schuldiner S. Vesicular monoamine transporters heterologously expressed in the yeast *Saccharomyces cerevisiae* display high-affinity tetrabenazine binding. *Biochim Biophys Acta* 1510: 426–441, 2001.
41. Yelin R, Steiner-Mordoch S, Aroeti B, Schuldiner S. Glycosylation of a vesicular monoamine transporter: a mutation in a conserved proline residue affects the activity, glycosylation, and localization of the transporter. *J Neurochem* 71: 2518–2527, 1998.
42. Yernool D, Boudker O, Jin Y, Gouaux E. Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. *Nature* 431: 811–818, 2004.

