2012

FUNCTIONAL AND BIOCHEMICAL CONSEQUENCES OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE HUMAN VESICULAR MONOAMINE TRANSPORTER 1 GENE (SLC18A1) By Sally Gamal Shukry, B.S.

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Richmond, Virginia
May, 2012

Acknowledgement

I would like to acknowledge several people who have been vital to my success. First and foremost, I would like to thank Dr. Stewart, who has taken on many roles starting with that of the mentor, the friend, the confidant and the guidance counselor. Secondly, I would like to thank Dr. Ahmed Khalifa, my husband and my best friend. His continued support and unyielding faith has been and will be the driving force behind all that I continue to achieve. I would also like to thank my family for their unwavering support and patience. I would also like to thank my committee members, Dr. Joseph Porter, Dr. Michael Fine and Dr. William Eggleston. Lastly, I would like to thank Dr. Krista Stenger and her lab for performing the western blots.

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List of Abbreviations

5HT: Serotonin

ACTH: Adrenocorticotropic Hormone

ANOVA: Analysis of Variance

ATP: Adenosine Triphosphate

ATPase: Adenosine triphosphatase

cDNA: Complementary Deoxyribonucleic acid

COS1: African Green Monkey Kidney Cells

CRH: Corticotropin Releasing Hormone

DAT: Dopamine Transporter

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

GI: Gastrointestinal

^{3}[H]5HT: Tritium labeled serotonin

hVMAT1: Human Vesicular Monoamine Transporter 1

IC_{50}: Half maximal inhibitory concentration

IgG: Immunoglobulin G

Ile: Isoleucine

KOH: Potassium Hydroxide

NET: Norepinephrine Transporter

PBS: Phosphate buffered saline

PMSF: Phenylmethylsulfonylfluoride

Pro: Proline

RT-PCR: Reverse Transcription Polymerase Chain Reaction

Ser: Serine

SERT: Serotonin Transporter

SH: Sucrose-Hepes

SLC18A1 : Solute Carrier Family 18, Member 1

SNP: Single Nucleotide Polymorphism

TBS: Tris-Buffered Saline

TBST: TBS + 0.1% Tween-20

Thr: Threonine

VMAT1: Vesicular Monoamine Transporter 1

VMAT2: Vesicular Monoamine Transporter 2

VMATs: Vesicular Monoamine Transporters

Abstract

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By Sally Gamal Shukry, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Major Advisor: Jennifer K. Stewart
Associate Professor and Graduate Director, Department of Biology

Single nucleotide polymorphisms (SNP) in the human VMAT1 gene (SLC18A1) have been associated with schizophrenia in three different populations: Han Chinese, Western European and Japanese. Effects of these mutations on transport function of the hVMAT1 protein have not been reported. The goal of this study was to investigate functional and biochemical differences in human VMAT1 proteins with a threonine or proline at amino acid position 4 (Thr4Pro) and a serine or threonine at position 98 (Ser98Thr). COS1 cells were transfected with variant SNPs

coding for 4Thr/98Ser, 4Pro/98Ser, or 4Thr98Thr. Western blotting demonstrated robust over expression of the genes and no differences in electrophoretic mobility of the proteins. Maximal transport of serotonin by the VMAT1 protein with 4Pro/98Ser was less than that of the 4Thr/98Ser or the 4Thr/98Thr. Response of the 4Pro/Ser98 to the VMAT inhibitor reserpine was lower than that of the 4Thr/98Thr. These findings suggest mechanisms for human VMAT1 links to schizophrenia.

Introduction

A Brief Background on Vesicular Monoamine Transporters

Vesicular monoamine transporters (VMATs) are 55 kD transport proteins that are embedded in the membranes of intracellular vesicles (5;27). VMAT proteins are members of the Solute Carrier gene family (SLC18) that transport monoamines such as norepinephrine, dopamine, and serotonin into storage vesicles to allow subsequent release of the vesicle contents into a synapse or other extracellular space (10). Two major VMATs, VMAT1 and VMAT2, are 60% identical in terms of their amino acid sequences. VMAT1 (known as the chromaffin granule amine transporter) is found in extraneural tissues including the chromaffin cells of the adrenal medulla and endocrine and paracrine cells of the GI tract. VMAT2 (known as the synaptic vesicular monoamine transporter) is primarily located in neuronal cells of the central, peripheral and enteric nervous systems (8;19). By accumulating newly synthesized neurotransmitter molecules and freshly returned neurotransmitters from the synapse, VMAT2 plays a critical role in the various signaling processes between monoamine neurons (18). VMAT1 serves a similar role in adrenal medulla and neuroendocrine cells.

Although VMAT2 is the major VMAT in human and rodent brain (8;19), Karen Brennan-Ashe of the Stewart lab at Virginia Commonwealth University detected VMAT1 mRNA in the mouse medulla oblongata with the reverse transcriptase polymerase chain

reaction (RT-PCR) (1). The novel finding of VMAT1 in human post mortem brain by Lohoff et al. with RT-PCR and Western Blots further increased interest in the localization of VMAT1 in the human brain (13). The highest levels of human VMAT1 were identified in the substantia nigra, amygdala and hippocampus (13).

A Look At How VMATs Work

Like plasma membrane transporters such as the dopamine transporter (DAT), serotonin transporter (SERT), and norepinepherine transporter (NET), VMATs display a similar size and topography with 12 transmembrane domains and both C-terminal and N-terminals tails in the vesicle's interior (11;17), and Figure 2). Vesicular monoamine transporters differ from other membrane transporters in several ways. Most importantly, VMAT utilizes a proton gradient energetically to transport substrates rather than the Na^+/K^+ gradient used by DAT, SERT, and NET (25). The proton gradient is established across the vesicular membrane, which is created by a vacuolar ATPase (not directly associated with the transporter) in its antiport activity, exchanging two protons for each monoamine taken into the vesicle (22). Once the first proton is bound to the transporter, a conformational change exposes a high affinity binding site. Binding of the second proton causes the monoamine to be transported into the vesicular space (26). During an action potential, vesicles move to the cell membrane and contents of the vesicle are discharged into the extracellular space by exocytosis (10).

The Link Between VMAT and Schizophrenia

Human VMAT1 is located on chromosome *8*. There are 484 annotated genes located on *8p 21-22*; many of which are proto-oncogenes and tumor-suppressor genes (24). This region of the genome also is one of the regions most implicated for susceptibility to schizophrenia and bipolar disorders. Roughly 8.5% of the genes contained on 8p are believed to contribute to neuropsychiatric disorders such as schizophrenia, autism, bipolar disorders and depression (12). They are also implicated in neurodegenerative disorders such as Parkinson's and Alzheimer's disease (24).

A number of single nucleotide polymorphisms (SNP) have been identified in the human *Vmat1* gene (SLC18A1), SNPs are DNA sequence variations that occur when a single nucleotide (A, T, G or C) differs among members of a species, and geneticists are particularly interested in whether SNPs in human genes are more common in individuals with a specific disorder.

Based on the *Vmat1* gene location on chromosome *8*, Bly tested the hypothesis that a VMAT1 SNP is associated with schizophrenia. Sequenced DNA from post-mortem brain tissue of schizophrenic Caucasians revealed a statistically significant association with a homozygous SNP in exon 3 of the *SLC18A1* gene. This single base change, A277C (numbering based on GenBank Accession # NM_003153.3), resulted in a single amino acid change in the primary sequence of VMAT1 that produced a proline rather than a threonine at amino acid 4. The CC genotype coding for the proline was observed in 21.4%

of the schizophrenic group compared to 2.6% in the control group (2). This finding was supported by identification of the same SNP linked to schizophrenia in a Han Chinese population (3). Additionally, two other laboratories have reported statistically significant associations of this and other SNPs of the *Vmat1* gene with either schizophrenia or bipolar disorder in populations of European and Japanese descent (14;20). Three SNPs in the coding region of the human *Vmat1* gene that have been associated with schizophrenia are listed in Table 1. A partial sequence of the VMAT1 cDNA NM_003053 illustrating the position of SNPs in the coding region is shown in Figure 1. The predicted structure of the human VMAT1 protein is shown in Figure 2.

Table 1. Single Nucleotide Polymorphisms In The Human VMAT1 gene SLC18A1

*SNP ID	+NM_003053.3 Bases & NP_003044.1 Amino Acids	SNP Alleles	Major SNP Allele	Major RNA→Amino Acid
rs2270641	A277C Thr4 Pro	T/G	T	A→Thr
rs2270637	G560C Ser98Thr	C/G	C	G→Ser
rs1390938	T674C Thr136Ile	A/G	G	C→Thr

*SNP ID from the NCBI SNP data base and + GenBank Accession Numbers

```
                  C
268 ATG CTC CGG ACC ATT CTG GAT GCT CCC CAG CGG TTG CTG AAG GAG GGG AGA GCG TCC CGG
     M   L   R  P/T  I   L   D   A   P   Q   R   L   L   K   E   G   R   A   S   R

328 CAG CTG GTG CTG GTG GTG GTA TTC GTC GCT TTG CTC CTG GAC AAC ATG CTG TTT ACT GTG
     Q   L   V   L   V   V   V   F   V   A   L   L   L   D   N   M   L   F   T   V

388 GTG GTG CCA ATT GTG CCC ACC TTC CTA TAT GAC ATG GAG TTC AAA GAA GTC AAC TCT TCT
     V   V   P   I   V   P   T   F   L   Y   D   M   E   F   K   E   V   N   S   S

448 CTG CAC CTC GGC CAT GCC GGA AGT TCC CCA CAT GCC CTC GCC TCT CCT GCC TTT TCC ACC
     L   H   L   G   H   A   G   S   S   P   H   A   L   A   S   P   A   F   S   T

                                                                   C
508 ATC TTC TCC TTC TTC AAC AAC AAC ACC GTG GCT GTT GAA GAA AGC GTA CCT AGT GGA ATA
     I   F   S   F   F   N   N   N   T   V   A   V   E   E   S   V   P S/T  G   I

568 GCA TGG ATG AAT GAC ACT GCC AGC ACC ATC CCA CCT CCA GCC ACT GAA GCC ATC TCA GCT
     A   W   M   N   D   T   A   S   T   I   P   P   P   A   T   E   A   I   S   A

                                                               T
628 CAT AAA AAC AAC TGC TTG CAA GGC ACA GGT TTC TTG GAG GAA GAG ACT ACC CGG GTC GGG
     H   K   N   N   C   L   Q   G   T   G   F   L   E   E   E  T/I  T   R   V   G

688 GTT CTG TTT GCT TCA AAG GCT GTG ATG CAA CTT CTG GTC AAC CCA TTC GTG GGC CCT CTC
     V   L   F   A   S   K   A   V   M   Q   L   L   V   N   P   F   V   G   P   L
```

Figure. 1. **Partial Sequence of NM_003053 (SLC18A1) with variants.** Numbers are initial base numbers of codons of VMAT1 mRNA (GenBank Accession # NM_003053. Letters below the codons are standard amino acid abbreviations. Polymorphisms are highlighted in yellow, and the alternate base is above the codon.

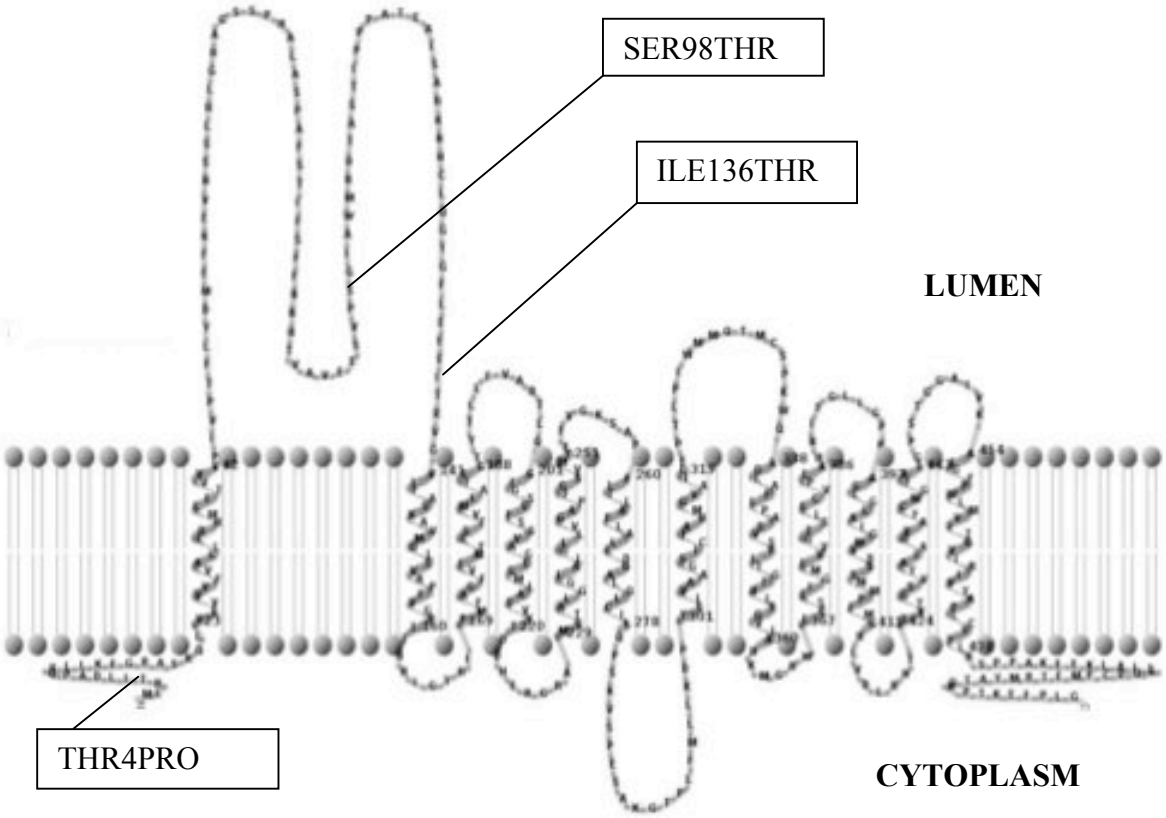

Figure 2. Predicted structure of the human VMAT1 protein. HMMTOP software (http://www.enzim.hu/hmmtop/html/document.html) was used to generate the structure from the translated protein sequence NP_003044.1 = protein coded by NM_003053 mRNA.

Evidence is compelling for association of these SNPs shown in Figure 1 with psychiatric disorders, butt there is no information on functional consequences of any of these SNPs. As shown in Figure 2, amino acid 4 is located in the N-terminal cytoplasmic tail of the transport protein, and amino acids 98 and 136 are located in the first intra-vesicular loop. Both cytoplasmic and intravesicular regions are postulated to interact with

substrates and inhibitors (16;23), but specific effects of the amino acid changes on VMAT1 transport activity are not known. The goal of this study was to address this question by creating specific mutations in the human VMAT1 gene, transfecting the genes into COS1 cells and evaluating effects on the transport activity of protein expressed in the cells. The initial reference gene used in this study coded for the haplotype Thr4 Ser98 Ile136. This haplotype was chosen as the reference based on genetic evidence that the alternate alleles are associated with schizophrenia or related disorders. The 4Pro and Thr98 are linked to schizophrenia (2;3;14;20), and the 136 Ile is associated with partial protection against bipolar disorder (13).

Materials and Methods

Cells

The COS-1 kidney fibroblast cell line was obtained from American Type Culture Collection (ATCC # 30-2002, Manassas, VA). Cells were maintained at 37° C with 5% CO_2 in DMEM medium (# 30-2002, ATCC) containing 4 mM L-glutamine, 4.5 g/L glucose and 1.5g/L sodium bicarbonate. COS-1 medium is supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin.

Site-directed mutagenesis

The VMAT1 clone with the reference sequence coding for 4Thr/98Ser/136 Ile was obtained from Origene - SC122643 in the pCMV6-Neo vector. Primers designed by our research team and used for mutagenesis by the VCU Molecular Laboratory are shown in Table 2. Variant genes were inserted into expression vectors, amplified in *E.coli (end A$^-$ and Rec A$^-$)* and the plasmids purified in megapreps by the VCU Molecular Biology Core. Incubations were at 30 º C as recommended by Origene. Full length sequencing by Operon (Huntsville, AL) verified all mutations and absence of recombination during cloning.

Table 2. Mutagenesis Primers

Amino Acid Change to		Sense (S)/Antisense (AS)
4 Pro	S	ATCACCATGCTCCGG*C*CCATTCTGGATGCTC/
	AS	GAGCATCCAGAATGG*G*CCGGAGCATGGTGAT.
98 Ser	S	GTTGAAGAAAGCGTACCTA*C*TGGAATAGCATGGATGAATG/
	AS	CATTCATCCATGCTATTCCA*G*TAGGTACGCTTTCTTCAAC.

Cell Transfection

To examine VMAT1 expression, COS-1 kidney fibroblasts were transfected with the VMAT1 cDNA according to the protocol of Finn et al. (9). Cells were grown to confluence in T-75 flasks (CoStar, Fisher Scientific) and fed 24 hours prior to electroporation. In preparation for electroporation and transfection, cells were rinsed with calcium-free/magnesium-free phosphate buffered saline (PBS) and detached with 0.25% trypsin and 0.03% EDTA. Cells were returned to medium containing serum to inactivate the trypsin and pelleted by centrifugation at 500 x g for 10 minutes at 4 °C. The cells were resuspended in 800 µl of pre-warmed PBS containing calcium and magnesium (Invitrogen). The cell suspension and 15 µg of DNA were transferred to a 0.4 cm gap cuvette in the gene Pulser II (Biorad, Hercules, CA) and electroporated at 0.4 kV and 975 µF. Cells were plated in T-75 flasks and incubated for three-4 days prior to preparation of membranes for transport assays or lysis for western analysis. The expression vector has a neomyocin resistance gene, and cells incorporating the plasmid were selected by growth in medium with geneticin (500 µg/ml).

Membrane Preparation

On the fourth day after electroporation, COS-1 cells from each T-75 flasks were rinsed with PBS, detached and pelleted with centrifugation as described above. The pellet was washed twice in PBS, centrifuged after each wash and resuspended in 200 µl of sucrose-Hepes buffer (SH) containing 0.32 M sucrose in 10 mM Hepes adjusted to pH 7.4 with 1 M KOH and supplemented with proteolytic inhibitors (2 ug/ml aprotinin and leupeptin 1 ug/ml pepstatin A and E-64, and 0.2 mM phenylmethylsulfonylfluoride (PMSF). The suspension was transferred to a 1.5 ml tube and homogenized by sonication at 4 pulses, 0.5 seconds per pulse, to yield approximately 3 Watts power. After the suspension was centrifuged at 500 x g for 10 minutes at 4 °C, the supernatant was immediately removed to a tube on ice. Total protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of the supernatant were stored at – 70 °C until assayed for VMAT activity.

Western Blotting

Membrane proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated for an hour at 24 ˚C in blocking solution containing Tris Buffered Saline (TBS) and 3% dry milk, and then incubated overnight at 4 C with primary goat anti-human VMAT1 antibody C-19 (#SC-7718, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in TBS + 0.1% Tween-20 (TBST). Blots were washed with TBST and incubated for an hour at 24 °C with 1:10,000 donkey

anti-goat IgG-horseradish peroxidase (Santa Cruz). The blots were visualized with enhanced chemiluminesence (Amersham, Biosciences, UK).

VMAT Uptake Assay

Each assay tube contained 50 μg of cell protein, 5 mM ATP, 0.25 mM ascorbic acid, and 20 nM radiolabeled serotonin (^{3}H-5HT) = 5-H[G-^{3}H]T creatine sulfate 27 *Ci* / mmol (American Radiolabeled Chemicals, Saint Louis, Mo), except in saturation experiments in which increasing concentrations of labeled + unlabeled serotonin were incubated to achieve saturation. Reactions were in a total volume of 200 μl of uptake buffer. Incubations were conducted at 27°C for 5 minutes, except in time course experiments or in saturation assays in which incubation was for 1.5 min to approximate initial velocity of transport. The reaction was terminated by addition of 1 ml cold SH buffer, rapidly filtered through 0.2 μm Supor 200 membranes (Pall/Gelman # 60300), and washed with twice with 1 ml of cold SH buffer in a Millipore sampling manifold. Filters were transferred to 10 ml of Scintisafe 50% Plus scintillation fluid (Fisher Scientific,) and radioactivity was measured with a Beckman LS6000IC scintillation counter.

Statistics

Statistical analyses were as follows: For comparisons of VMAT1 activity at various time points, a split-plot two-way analysis of variance (ANOVA with groups as between-subjects factor and time as the within-subjects factor) and Newman Keuls' post-

hoc tests were used. For comparison of activity measured at 5 min a one-way ANOVA and Newman Keul's post hoc was used. For transport kinetics and inhibition curves, best fit non-linear regressions were performed on data from 3 different transfections of each variant, and significant differences were determined from the 95% confidence limits of the regressions. ANOVAs and non-linear regressions were performed with GraphPad Prism 5. (GraphPad Software, Inc., La Jolla, CA).

Results

Expression of hVMAT1 Variants in Transfected Cells

Western blotting confirmed expression of hVMAT1 variants in COS1 cells transfected with hVMAT1 gene variants (Fig. 3). As shown in Fig. 3, loading of only 2 µg of total protein was sufficient to observe marked immunoreactiveVMAT1 protein bands at 63 and 29 Kd . There were no differences in electroporetic mobility of the proteins on gels. The translated hVMAT1 protein sequence NP_003044.1 consists of 525 amino acids and a molecular size of 56 Kd (calculated with the Science Gateway Protein Molecular Weight Calculator at http://www.sciencegateway.org/tools/proteinmw.htp), but the major imunoreactive hVMAT1 proteins observed were 63 and 29 Kd. The 63 Kd size is consistent with evidence for phosphorylation and glycosylation of VMATs (7). The 29 Kd protein is hypothesized to be a truncated form lacking transport activity, as vesicular transporters usually have 12 membrane-spanning domains that yield molecular weights greater than 50 Kd (6).

Time-course of Serotonin Uptake by hVMAT1 Variants

The time course of radiolabeled serotonin uptake by protein extracts from cells transfected with hVMAT1-a (Thr4 Ser98 Ile136) or hVMAT-98Thr (Thr4 Thr98 Ile136) or hVMAT-1 4Pro (Pro4 Ser98 Ile136) increased steadily and was approximately linear

over at least 5 min (Fig. 4). Serotonin uptake by protein from wild type cells did not increase over time. There were no statistical differences in the uptake of serotonin by hVMAT1a and hVMAT-98Thr over 15 minutes, but the time course of radiolabeled serotonin uptake by hVMAT1- 4Pro differed significantly from both hVMAT1-a and hVMAT-98Thr. A quantitative comparison of serotonin uptake measured at 5 min is shown in Fig. 5.

Inhibition of hVMAT1 Variants by Reserpine

The first studies of response to inhibitors were performed with the VMAT inhibitor reserpine, and these studies were used to establish methodology for subsequent assays. Preliminary inhibition curves were performed with extracts from 7 transfections of each of two variants. These data (not shown) identified occasional spurious results demonstrating the necessity for additional repetition to verify the reproducibility of results.

Fig. 6 illustrates average inhibition curves for effects of reserpine on radiolabeled serotonin uptake by the variant protein cell extracts. The average IC_{50} (Table 3) of reserpine inhibition was significantly greater for the hVMAT with a 4Pro/Ser98 (Pro4 Ser98 Ile136) than for the 4Thr/98Thr (Thr4 Thr98 Ile136) ($P < 0.05$). Reserpine inhibition of hVMAT1-a (Thr4 Ser98 Ile136) and hVMAT-98Thr (Thr4 Thr98 136 Ile) did not differ significantly.

Inhibition of Serotonin Uptake by hVMAT1 Variants by Dopamine

Dopamine inhibition of serotonin uptake by protein extracts from cells transfected with expression vectors carrying the three different haplotypes (Fig. 7 and Table 3) did not differ significantly.

Saturation Kinetics of Serotonin Uptake by Human VMAT1 Variants

Saturation studies were performed at 1.5 min in an attempt to achieve initial velocity of transport. As shown in Fig. 8 and Table 4, the transport Vmax of the hVMAT-1a (Thr4 Ser98 Ile136) and hVMAT-98Thr (Thr4 Thr98 Ile136) variants was greater than that of hVMAT-4Pro (Thr4 Ser98 Ile136). Although the transport Km of the 4Pro/Ser98 variant protein was slightly lower than that of the other variants, the difference was not significant (Table 4). In time course studies performed with 20 nM radiolabeled serotonin the variants hVMAT-1a (Thr4 Ser98 Ile136) and hVMAT-98Thr (Thr4 Thr98 Ile136) did not differ significantly, and with saturating concentrations of radiolabelled serotonin, the transport Vmax of these variants also did not differ significantly (Fig. 8 and Table 4).

Discussion

This study provides new information on the functional and biochemical effects of single nucleotide polymorphisms in the human VMAT1 gene that have been linked to psychiatric disorders. This information is anticipated to provide clues to mechanisms that account for linkage of these SNPs to schizophrenia and other psychiatric disorders.

Genetic linkage studies performed by several laboratories suggest that the proline at position 4 is associated with increased susceptibility to schizophrenia in Han Chinese and western European populations (2;3;14). The results of the present study indicate that the hVMAT-1 4Pro/98Ser haplotype is associated with a decrease in transport activity compared with other haplotypes in this study (Fig. 4 and 5), This finding is consistent with evidence that reduced storage of monoamines in the brain and adrenal medulla and potential disruption of regulatory mechanisms of adrenal-hypothalamic-pituitary interactions will increase susceptibility to mood and behavioral disorders and potential changes in cognitive functions (15). The transport Km of this haplotype was slightly but not significantly lower than that of the other variants. It appears unlikely, therefore, that a higher affinity for substrate could compensate for the reduced maximal function of the 4Pro variant. Furthermore, there were no detectable differences among the variants in response to dopamine inhibition of serotonin uptake.

There were no significant differences in transport function between the 4Thr/98Thr and 4Thr/98Ser haplotypes. Although the SNP coding for either a threonine 98 or a serine

98 has been linked to schizophrenia in both Chinese and Japanese populations (3;20), it is a different allele in each population that is more frequent in schizophrenic patients. This raises the possibility that other factors in combination with a specific 98 amino acid may increase susceptibility to schizophrenia. The more prominent effect on transport of the SNP coding for a 4 proline may be because of its location in the cytoplasmic N-terminus. Additionally, there is evidence for phosphorylation for VMATs (7), and whereas both threonine and serine can be phosphorylated, proline is not a substrate for phosphorylation.

It is clear that changes in VMAT1 in brain could alter storage of monoamine neurotransmitters, but an additional hypothesis linking changes in VMAT1 to mood and behavioral changes is based on the function of VMAT1 in storing epinephrine in vesicles in the adrenal medulla. These vesicles are primed at the plasma membranes to be exocytosed when triggered. VMAT-1 is the major vesicular monoamine transporter that functions in adrenal medullary storage and regulated release of catecholamines, particularly epinephrine, in rodents as well as humans (7). Decreased function of VMAT-1 in the adrenal medulla will reduce storage and release of adrenal catecholamines, which may produce a deficiency of the rapid mechanism responsible for alleviating hypoglycemia during fasting or stress (4). Assuming that the 4Pro haplotype would cause less epinephrine to be stored and released, less epinephrine will be available to stimulate mechanisms that alleviate hypoglycemia. Persistent hypoglycemia during epinephrine deficiency will stimulate increased release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and corticosteroids, and glucagon. Increased

production of CRH and continuing elevation of plasma corticosteroids increase susceptibility to depression and anxiety (15) and alter cognitive neural pathways in the hippocampus and striatum (15;21)

In summary, this study suggests that a proline at position 4 in hVMAT1 causes a modest reduction in VMAT1 transport function and alters its response to the inhibitor reserpine. In contrast, the exchange of a threonine for a serine at position 98 in the protein appears to have little effect on transporter function. Additional study is needed to clarify the functional consequences of the Thr136Ile polymorphism in hVMAT1 and combinations of amino acid changes at the 4, 98, and 136 positions.

Results Figures

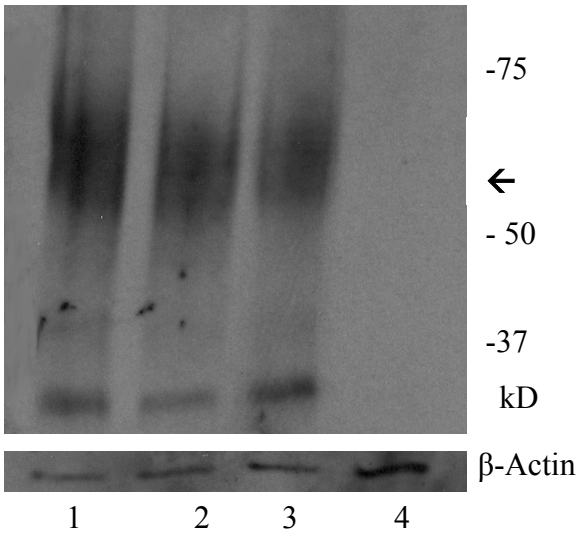

Figure 3. Immunoreactive human VMAT-1 assayed by western blotting. Beta actin loading controls are shown below each lane. Total protein (2 μg per lane) was separated by SDS-PAGE on 10 % gels. Lane 1= COS1 cells+hVMAT1a (4Thr/98Ser/136Ile); 2 = COS1+hVMAT1 (4Thr/98Thr/136Ile); 3 = COS1+hVMAT1 (4Pro/98Ser/136Ile); 4 = COS WT cells. The arrow indicates a 63 kD protein hypothesized to be the major active hVMAT1 protein.

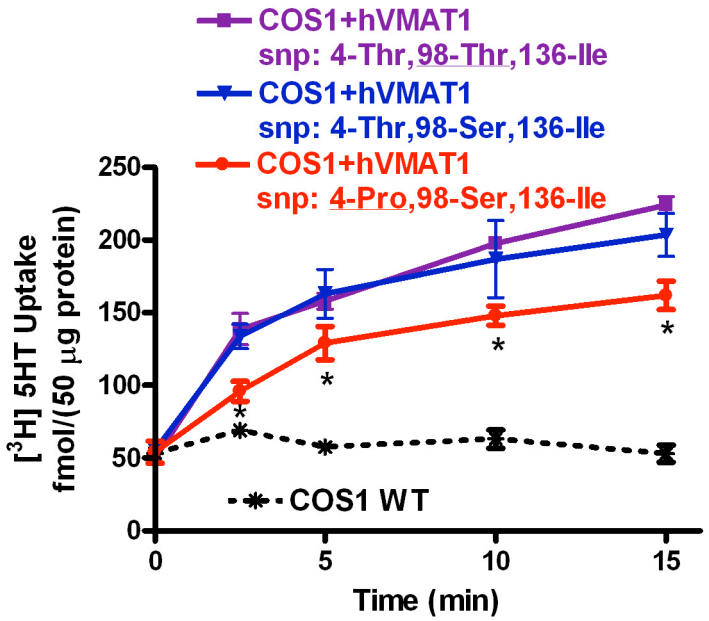

Figure 4. **Time course of serotonin uptake by human VMAT1 variants.** Uptake of radiolabeled serotonin ([^{3}H] 5HT) by protein extracts from wild type (WT) COS1 cells or cells transfected with expression vectors carrying human VMAT1 with polymorphisms coding for a threonine or serine at amino acid 98 and threonine or proline at amino acid 4. The data represent the mean ± S.E. of 3 transfections for each variant. Uptake of each variant was significantly greater than that of WT cells at each time point. Uptake by hVMAT1 with a 4Pro was significantly less than that of the hVMAT1a98Ser and hVMAT98Thr by two-way ANOVA and Newman Keuls' post-hoc test (* $P < 0.01$).

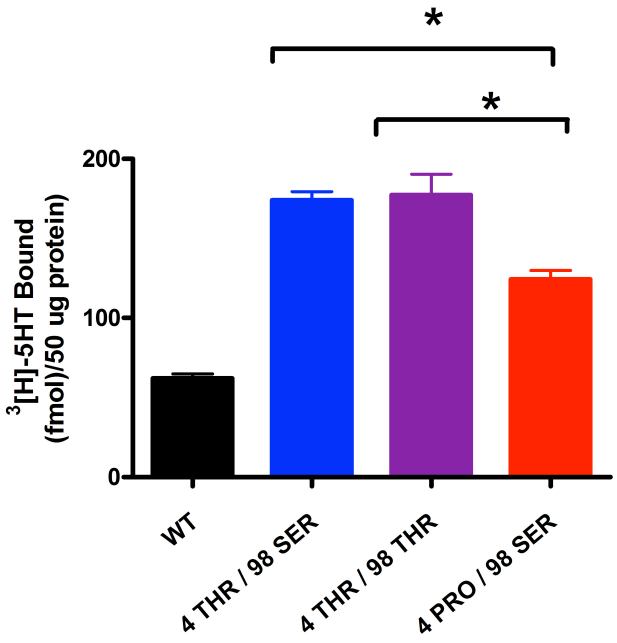

Figure 5. **Quantitative comparison of serotonin uptake at 5 minutes by human VMAT1 variants.** Comparison of radiolabeled serotonin ([^{3}H] 5HT) by protein extracts from wild type (WT) COS1 cells and cells transfected with expression vectors carrying human VMAT1 with polymorphisms coding for a threonine or serine at amino acid 98 and a threonine or proline at amino acid 4. Each bar represents the mean ± S.E. of 9-12 replicates from 3-7 transfections for each variant measured at 5 min incubation. All are significantly different from wild type by one-way ANOVA and Newman Keuls' post-hoc test. * $P < 0.01$ compared to 4 PRO/98SER.

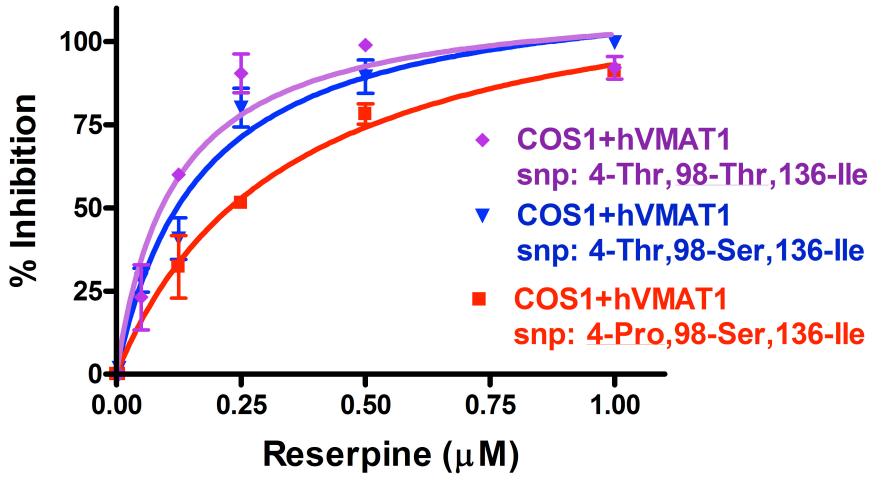

Figure 6. Reserpine inhibition of serotonin uptake by human VMAT1 variants. Protein extracts from cells transfected with expression vectors carrying human VMAT1 DNA coding for the different haplotypes were incubated for 5 minutes with vehicle or 5-6 concentrations of reserpine and tritium labeled serotonin. Each curve represents the average of 3-5 curves run on 3-5 transfections of each variant with wild type values subtracted. The average IC_{50} (Table 3) of reserpine inhibition was significantly greater for the hVMAT1 with a 4Pro/Ser98 (Pro4 Ser98 Ile136) than for the 4Thr/98Thr (Thr4 Thr98 Ile136) based on 95% confidence limits of the best fit non-linear regressions.

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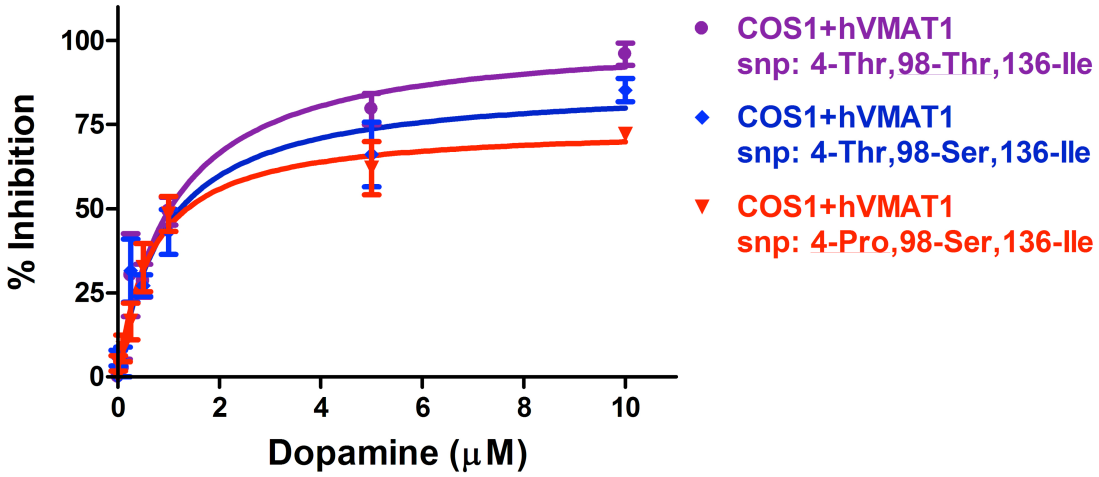

Figure 7. Dopamine inhibition of serotonin uptake by human VMAT1 variants. Protein extracts from cells transfected with expression vectors carrying human VMAT1 DNA coding for the different haplotypes were incubated for 5 minutes with vehicle or 5-6 concentrations of dopamine and tritium labeled serotonin. Wild type values were subtracted from each point. The average IC_{50} (Table 3) of dopamine inhibition of serotonin uptake did not differ significantly among the three protein variants.

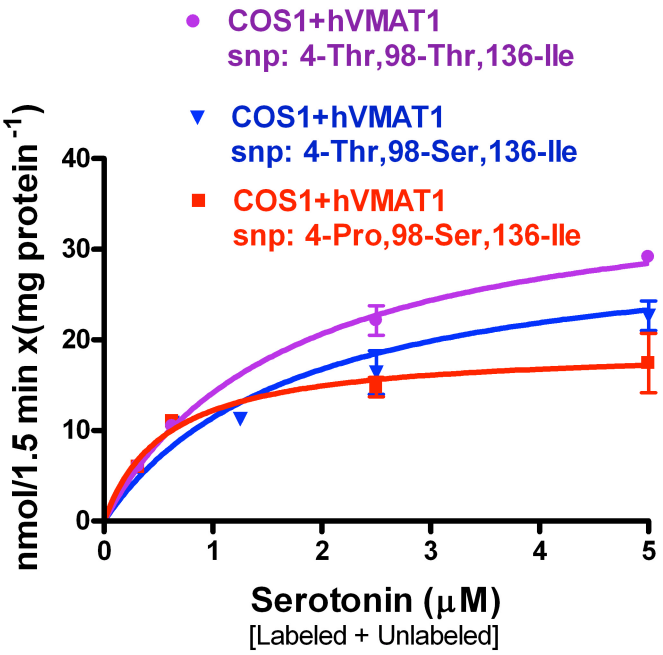

Figure 8. Saturation kinetics of serotonin uptake by human VMAT1 variants. Curves illustrate uptake of 1.0:1.2 radiolabeled:unlabeled serotonin over 1.5 min by protein extracts from COS1 cells transfected with expression vectors carrying human VMAT1 with polymorphisms coding for a threonine or serine at amino acid 98 and threonine or proline at amino acid 4. Each data point represents the mean ± S.E. of 3-5 replicates from 3 transfections per variant with wild type values subtracted. Transport Km and Vmax values are in Table 4.

Table 3. Inhibition of Human VMAT1 Variant Uptake of Serotonin

hVMAT1 Haplotype	Reserpine IC_{50} (nM) Mean ± S.E	Dopamine IC_{50} (μM) Mean ± S.E.
4Thr/98Ser/	169 ± 29	0.92 ± 0.22
4Thr/98Thr	116 ± 31	1.07 ± 0.21
4Pro/98Ser	271 ± 34*	0.70 ± 0.15

*$P \leq 0.05$ compared to the IC_{50} of the 4Thr/98Thr haplotype based on 95% confidence limits of the best fit non-linear regression.

Table 4. Saturation Kinetics of Serotonin Transport
by Human VMAT1 Variants

hVMAT1 Haplotype	Km µM Mean ± S.E	Vmax nmol/1.5 min(mg protein^{-1}) Mean ± S.E
4Thr/98Ser/	1.76 ± 0.48	31.53 ± 3.09
4Thr/98Thr	1.68 ± 0.48	38.00 ± 3.53
4Pro/98Ser	0.57 ± 0.22	19.17 ± 2.13*

*$P \leq 0.05$ compared to 4 Thr/98Ser and 4Thr98Thr based on 95% confidence limits of the best fit non-linear regression.

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