MUTATION OF THE VESICULAR MONOAMINE TRANSPORTER-1 GENE ALTERS ITS PROTEIN PRODUCT

Abena Watson-Siriboe
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Biology Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/77
This is to certify that the thesis prepared by Abena B. Watson-Siriboe entitled MUTATION OF THE VESICULAR MONOAMINE TRANSPORTER-1 GENE ALTERS ITS PROTEIN PRODUCT has been approved by his or her committee as satisfactory completion of the thesis or dissertation requirement for the degree of Masters of Science.

Director of Thesis, Jennifer K. Stewart PhD., College of Humanities and Sciences

Committee Member, John J. Ryan PhD., College of Humanities and Sciences

Committee Member, Joseph H. Porter PhD., College of Humanities and Sciences

Committee Member, Wan-Ling Chiu PhD., College of Humanities and Sciences

Committee Member, Michael L. Fine PhD., College of Humanities and Sciences

Fred M. Hawkridge, Interim Dean, College of Humanities and Sciences

Dr. F. Douglas Boudinot, Dean of the School of Graduate Studies

[Click here and type the Month, Day and Year this page was signed.]
MUTATION OF THE VESICULAR MONOAMINE TRANSPORTER-1 GENE
ALTERS ITS PROTEIN PRODUCT

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

by

ABENA BARBARA WATSON-SIRIBOE
BACHELORS OF SCIENCE, UNIVERSITY OF CALIFORNIA, RIVERSIDE, 2006
MASTERS OF SCIENCE, VIRGINIA COMMONWEALTH UNIVERSITY, 2010

Director: JENNIFER K. STEWART, PHD
ASSOCIATE PROFESSOR, DEPARTMENT OF BIOLOGY

Virginia Commonwealth University
Richmond, Virginia
May 2010
Acknowledgement

The author would like to acknowledge several people who have been integral to her success. First and foremost, I would like to thank Dr. Stewart. Dr. Stewart has not only been a great advisor, but has been integral in further molding a laid back budding scientist. But, she has been a great confidant, when things became a bit overwhelming. Secondly, I would like to thank my family. They were not only supportive of my move across the country, but patient as they watched my growth. I would also like to thank my committee; Dr. John Ryan, Dr. Joseph Porter, Dr. Michael Fine and Dr. Wan-Ling Chiu. I would like to give a very special thank you to the Chiu lab for not only teaching me new techniques, but for the use of their lab. Last but not least, many thanks is given the Dr. Krista Stenger and her lab for performing the western blots.
# Table of Contents

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
</tr>
<tr>
<td>List of Tables</td>
</tr>
<tr>
<td>List of Figures</td>
</tr>
<tr>
<td>1 Introduction</td>
</tr>
<tr>
<td>A Brief Review: Vesicular Monoamine Transporters</td>
</tr>
<tr>
<td>The Schizophrenia Conundrum</td>
</tr>
<tr>
<td>Linkage of VMAT1 to Schizophrenia</td>
</tr>
<tr>
<td>2 Materials and Methods</td>
</tr>
<tr>
<td>Cloning of hVMAT1 from Human Brain and Adrenal RNA</td>
</tr>
<tr>
<td>Primers</td>
</tr>
<tr>
<td>Not I Digestion of pCMV6-XL5 Vector, Ligation into pCMV6-Neo Vector and Electrophoresis</td>
</tr>
<tr>
<td>DNA Isolation from Agarose Gels</td>
</tr>
<tr>
<td>DNA Ligation and E.Coli Transformation</td>
</tr>
<tr>
<td>Plasmid Midi Prep and Determination of Insert Orientation</td>
</tr>
<tr>
<td>Site Directed Mutagenesis of hVMAT1 in Neomycin Vector</td>
</tr>
</tbody>
</table>

iii
Cells .............................................................................................................14

Cell Transfection .........................................................................................15

Membrane Preparation ................................................................................15

Western Blotting ..........................................................................................16

VMAT Uptake Assay ..................................................................................17

3 Results ..............................................................................................................18

Site Directed Mutagenesis of hVMAT1-pCMV6-Neo ..................................18

Creation of Stably Transfected Cell Lines ..................................................18

Analysis of Serotonin Binding Activity .......................................................20

4 Discussion ........................................................................................................20

Figures for Results ..........................................................................................23

References .........................................................................................................31
List of Tables

Table 1: Reverse Transcriptase Polymerase Chain Reaction Primers. ..........................7
Table 2: Mutagenesis and Sequencing Primers. ................................................................14
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Reverse Transcriptase Polymerase Chain Reaction in Human Adrenal and Brain samples</td>
<td>9</td>
</tr>
<tr>
<td>Figure 3</td>
<td>XMN-1 Digestion of hVMAT1-pCMV6-Neo to Determine Insert Orientation</td>
<td>13</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Site Directed Mutagenesis of VMAT 1 sequences</td>
<td>23</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Sequence of hVMAT1 DNA Obtained from Origene (A277C)</td>
<td>24</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Sequence of hVMAT1 DNA Subject to Site Directed Mutagenesis (VMAT)</td>
<td>25</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Expression of VMAT1 Protein in CHO-K1 Transfected Cells</td>
<td>26</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Expression of VMAT1 Protein in CHO-K1 Transfected Cells</td>
<td>27</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Expression of hVMAT1 in Transfected Cells</td>
<td>28</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Binding of Serotonin to Transfected hVMAT1 Protein</td>
<td>29</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Serotonin binding activity in Transiently Transfected COS-1 Cells</td>
<td>30</td>
</tr>
</tbody>
</table>
The vesicular monoamine transporter 1 (VMAT1) is essential for storage of monoamines, such as epinephrine and serotonin, in secretory vesicles of neuroendocrine cells. Recently the VMAT1 protein was detected in human and mouse brain, and mutations of the VMAT1 gene at single DNA nucleotides (single nucleotide polymorphisms or SNPs) were associated with schizophrenia. In this study, Chinese hamster ovarian cells were stably transfected with either human VMAT1 DNA (GenBank: #NM_003053.1) or DNA with the Thr4Pro SNP, which results in a threonine to proline change in amino acid number 4 of the VMAT1 protein. Western blot analysis revealed that cells with the SNP produced immunoreactive human VMAT1 proteins of altered molecular size, suggesting that SNP Thr4Pro modifies either folding or processing of the VMAT1 protein. This finding is the first evidence for biochemical consequences of a mutation in the human VMAT1 gene.
Introduction

A Brief Review of Vesicular Monoamine Transporters

The Solute Carrier gene family (SLC) consists of proteins that exhibit transporter function. Five SLC families are responsible for the transport of neurotransmitters, biogenic amines, ammonium and choline (9). Vesicular neurotransmitter transporters play a role in the proper function of neural processes by regulating the storage and release of classic neurotransmitters such as norepinephrine, dopamine, and serotonin. (17) These transporters act by sequestering neurotransmitters into subcellular compartments, such as vesicles, resulting in a regulation of the transmitter signal as well as protection against the cytotoxic effects of such compounds. Four types of vesicular neurotransmitter transporters have been identified: monoamine (VMAT), acetylcholine (VAcHT), glutamate (VGlUT) and GABA (VGAT) transporters (6). These transporters share several characteristics, but the VMATs are of particular interest in this study.

The vesicular monoamine transporters (VMATs) are part of the SLC18 gene family of vesicular amine transporters. Two isoforms have been identified, VMAT1 and VMAT2, which share a 60% sequence identity (5). Vesicular monoamine transporters are responsible for the sequestration of biogenic amines such as dopamine and norepinephrine within neuronal and endocrine cells (1). The structure of these transporters is characterized by twelve transmembrane domains resulting in a major 55 kDa molecule.(5). VMAT 1 has several substrates including dopamine, serotonin, norepinephrine and epinephrine. VMAT 2 shares the same substrates but binds histamine with higher affinity (5).
A vacuolar ATPase not directly associated with the transporter is responsible for producing a proton gradient across the vesicular membrane (5; 19). This proton gradient is utilized by the VMAT’s antiporter activity, exchanging two protons for each monoamine taken into the vesicle (19). Binding of the first proton to the transporter results in a conformational change, exposing a high affinity binding site. Binding of the second proton results in the transport of the monoamine into the vesicular space (24). During an action potential, contents of the vesicle are discharged into the extracellular space by exocytosis (9).

Although the VMATs share structural similarities, it was initially believed that they were located in different anatomical locations. Vesicular monoamine transporter 2 was initially identified in neurons of the brain and peripheral nerve endings, whereas; VMAT 1 is associated with the adrenal medulla and neuroendocrine cells (5). Karen Brennan of the Stewart lab (VCU) detected VMAT1 within the mouse medulla/pons by reverse-transcriptase polymerase chain reaction (RT-PCR). This novel finding is further complemented by the detection of VMAT1 in human postmortem brain tissue by both Western blots and real-time polymerase chain reaction (12). Highest levels of human VMAT1 were identified in the substantia nigra, amygdala and hippocampus (13). The novel discovery of VMAT1 in the human brain does not make the linkage of mutations in the transporter’s gene to bipolar disorder and schizophrenia surprising.
Schizophrenia is a multi-faceted neuropsychiatric disorder that affects 1% of the global population (16). The disorder is characterized by deteriorations in behavior, cognition and emotions (1). Symptoms of this disorder include: delusions, paranoid presentation and impaired memory (20). No single factor has been linked to susceptibility to developing the disorder. Combinations of environmental and genetic factors have been thought to increase susceptibility. Factors such as maternal infection and nutritional deficiency have been linked to susceptibility as well as increased paternal age and fetal hypoxia (22). The most commonly supported factors are those that affect neurodevelopment and neurotransmitter regulation.

The gene “Disrupted in Schizophrenia 1” (DISC1) has been linked to several psychiatric disorders including bipolar disorder, autism and schizophrenia (23). DISC1 has many protein partners and often acts as a precursor to several pathways. The protein is highly expressed during development and is associated with several processes such as synaptic plasticity, neural migration and acting as a cytoskeletal scaffold protein (3; 10). The protein also has been identified at synapses. However, its role serves in the etiology of schizophrenia is unknown.

The prevailing hypothesis for the etiology of schizophrenia focuses on dopamine, which functions in motor activity, attention, stress and reward seeking behavior (14). Dysregulation of dopamine, in a regionally specific manner, may be linked to symptoms of schizophrenia such as psychosis. Upregulation of dopaminergic receptors has been hypothesized to play a large role in the dysregulation of this neurotransmitter (11). Several
genes have been identified that play a role in dopamine dysregulation; however, these genes are rare and fail to account for a majority of schizophrenia cases.

Recent studies also implicate dysregulation of serotonin and specific serotonin receptors in schizophrenia. For example, the use of the drug iloperidone, an antagonist of both dopamine and serotonin receptors (15), has been approved by the FDA for treatment of schizophrenia.

Schizophrenia has a non-Mendelian mode of inheritance (8). Although multiple susceptibility genes have been identified to have links to schizophrenia, these genes each have a small effect on the susceptibility to the disorder (8). Alterations in genes such as DISC1 result in a several fold increase in risk of developing schizophrenia, but no single gene has been identified to definitively cause the disease upon mutation or deletion.

*Linkage of VMAT1 to Schizophrenia*

Chromosome 8 of the human genome is one of the best supported regions for susceptibility for schizophrenia and bipolar disorders. Approximately 8.5% of the genes contained in 8p are involved in cerebral development, plasticity and maintenance (21). Of the many genes on chromosome 8, one is of particular interest, SLC18A1 or the human VMAT1 gene.

Several single nucleotide polymorphisms have been identified in this gene. Sequenced DNA from schizophrenic Caucasian post-mortem brain tissue revealed a missense homozygous mutation in the SLC18A1. This single base mutation, A277C, resulted in a single amino acid change in the protein sequence conferring a threonine to
proline change at amino acid residue 4. This phenomena was seen in 21.4% of the schizophrenic group compared to 2.6% in the control group (2). The first time VMAT1 was associated with susceptibility to schizophrenia. This finding was supported by the identification of the same SNP linked to schizophrenia in a subset of the Han Chinese population (4). Furthermore, two other laboratories have reported associations of other mutations of the VMAT-1 gene with either schizophrenia or bipolar disorder in populations of European and Japanese descent (1; 18). Specific effects of these single nucleotide polymorphisms on expression and biochemical properties of the human VMAT1 protein are not known.

The goals of this study were: (1) To stably transfect a cell line with expression vectors containing wild type human VMAT1 DNA or human VMAT1 DNA with the A277C mutation, and (2) To investigate effects of the A277C mutation on VMAT1 protein produced by the cells, specifically effects on immunoreactive protein observed on western blots and on the protein’s transport activity. Investigation of biochemical consequences of VMAT1 mutations linked to schizophrenia may give further insight into the etiology of this complex disorder.
Materials and Methods

Cloning VMAT1 from human brain and adrenal RNA

The initial procedure was to reverse transcribe human brain and adrenal VMAT1 RNA to VMAT1 cDNA and amplify with PCR to obtain sufficient quantities of the DNA for cloning and transfection into cells. Human brain and adrenal RNA were obtained from Stratagene (La Jolla, CA 540133, 540005). Three µg of each sample RNA were reverse transcribed with the Superscript™ Preamplification System (Invitrogen, Carlsbad, CA). Each reaction contained 2µl of the following 10X stock solutions: 0.5 µg oligo dT primers, 25 mM MgCl2, 0.1M DTT and 10X PCR buffer containing 20mM Tris-HCl and 50 mM KCl. One µl of 10mM dNTP was added and enough DEPC water to result in a final volume of 21 µl. Reactants were incubated for 5 min at 42° C prior to addition of 1 µl Superscript RT II (200 U/µl) to sample tubes. Reactants were incubated for an additional 50 min at 42° C in a thermocycler. The reaction was terminated by incubating tubes at 72° C for 15 min then held at 4° C until placement on ice. Residual RNA was degraded using 2 U of RNase H (GibcoBRL) and incubated at 37° C for 20 min

Polymerase Chain Reaction (PCR)

Each PCR reaction contained 6 µl of RT product, 10 µl PCR buffer, 3 µl 50mM MgCl2, 2 µl 10 mM dNTP mix, 0.5 µl Taq DNA Polymerase (5 U/µl), 70.5 µl DEPC treated water, 8.0 µl primer mix (forward and reverse primers, 0.05 nmol/ul each). The cDNA was amplified for 40 cycles for the target and 30 cycles for β-actin (15 sec at 94 °C, 15 sec at 56 °C and 30 sec at 72 °C). The reaction was held at 72 °C for 5 min, then at 4
°C until samples were stored at -20 °C. PCR for β-actin was performed as a control to determine the efficacy of the RT-PCR reaction.

**Primers**

PCR primers for RT-PCR were created using the Primer 3 program ([http://frodo.wi.mit.edu/primer3/](http://frodo.wi.mit.edu/primer3/)) and ordered from Invitrogen (Carlsbad, CA). Primers were designed to span at least 100 bp past the beginning and end of the target sequences (Table 1). PCR product was isolated by size by gel electrophoresis containing 1% agarose, 1X TAE buffer (Invitrogen Carlsbad, CA) and 0.5 µl of Ethidium Bromide (10mg/ml). Electrophoresis was run for 0.5 hours at 90 V. Human VMAT1 band was expected at 1762 bp and human β-actin at 208 bp. Gels were visualized and photographed with the BioDoc-It Imaging System (UVP, LLC Upland, CA). RT-PCR product was expected to produce a band size of approximately 1762 bp upon analysis by gel electrophoresis.

**Table 1. Reverse Transcriptase Polymerase Chain Reaction Primers**

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Expected Size Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVMAT1</td>
<td>AAGTGAGTTTCTCCTCTGG</td>
<td>GTCCCAGGGAAAGAGGTGGT</td>
<td>1762</td>
</tr>
<tr>
<td>β-actin</td>
<td>GCCACGGCTGTCTTC</td>
<td>GTTGCGTACAGGTCTTTGC</td>
<td>208</td>
</tr>
</tbody>
</table>

As shown in Figures 1 and 2, RT-PCR of neither brain nor adrenal produced product that could be isolated from the gel for cloning. Bands at the bottom of the gel represent unused primers. β-actin controls for both adrenal (lane 6) and brain (lane 8)
samples produced robust bands at 208 bp (Figure 1). DNA in lanes 3 and 5 appears to be represent many amplified fragments, suggesting a problem with the primers chosen for the RT reaction.

Figure 1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). AD = adrenal; BR = brain; No RT = reactions omitting the reverse transcriptase enzyme. Expected positions of hVMAT1 and β-actin products are shown on the right. Unused primers are at the bottom of No RT and No RNA lanes. The product is lane 7 may reflect primer dimers.

When the reaction was repeated with additional RNA samples and with 40 cycles of amplification the results did not improve. Figure. 2 demonstrates the best of these results. A faint band of approximately 1700 bp was observed in the brain sample. However, the yield for the reaction was too low for cloning purposes.
Figure 2. Reverse Transcriptase Polymerase Chain Reaction in Human Adrenal and Brain samples. RT-PCR products from human brain and adrenal RNA are shown. AD= human adrenal gland. BR= human brain No RT= no reverse transcriptase. The expected position of hVMAT1 product is shown on the right. Bands at the bottom of all lanes may reflect unused primers.

Due to the difficulty in obtaining human VMAT1 cDNA from RNA, the human VMAT1 sequence was purchased from Origene (Rockville, MD) in the pCMV6-XL5 vector, which also contained the ampicillin resistance gene. In order to produce a stably transfected mammalian cell line, the VMAT1 cDNA insert had to be transferred to a vector containing both ampicillin and neomycin resistance as described below.
Transfer of hVMAT1 from pCMV6-XL5 to pCMV6-Neo Vector

SLC18A1 (h-VMAT-1) in a pCMV6-XL5 vector was purchased from Origene (Rockville, MD SC122643). The plasmid was amplified in E. coli DH5α and purified with a midi-prep protocol by the Molecular Biology Core Laboratory (Virginia Commonwealth University), yielding 100 µl of product containing 2.623 µg/µl of plasmid. One µg of plasmid DNA was digested with Not I (Invitrogen Carlsbad, CA). A portion of the sample (1µl) was diluted to a concentration of 0.5 µg/µl in T10-E0.1 buffer (10mM TrisHcl and 0.1mM EDTA, pH 8.0), and 1µg of vector containing the insert was digested with Not I (Invitrogen Carlsbad, CA) according to manufacturer directions. Not I digestion product was separated by gel electrophoresis on a gel containing 1% agarose, 1X TAE buffer (Invitrogen). A 5 µl aliquot of digestion product and a 1 kb ladder were loaded into the gel and run at 90 V for approximately 0.5 hrs. Two resultant bands were identified, the empty vector (approximately 5800 bp) and insert (approximately 2770 bp).

Upon imaging, the target bands were identified and excised from the gel. Excised band weight ranged from 47-48 mg. Insert DNA was isolated from the excised band with the QIAquick gel extraction kit (Qiagen Valencia, CA) by the manufacturer’s protocol and dissolved in T10-E0.1 buffer. The resultant concentration was measured with the NanoDrop (SID-10135606, Thermo Scientific).

DNA ligation and E. Coli transformation

Isolated insert was ligated into the pCMV6-Neo vector for the purpose of stable transfection. The ligation reaction consisted of : 1 µl 10X ligation buffer, 1ul pCMV6-Neo
vector, 2 µl purified insert, 2 µl T4 ligase (New England Biolabs Beverley, MA) and ultrapure water up to a final volume of 10µl. The ligation was allowed to react for one hour at 37 °C. Transformation of DH5α E. coli cells was performed with the heat shock method (50 µl of E. Coli cell culture was combined with 10 µl of ligation mix and allowed to incubate on ice for 20 min. The mixture was then incubated at 37° C for 4 min, then allowed to grow for 1.5 hours at 37° C while shaking. The resultant culture was transferred to an agar plate containing 100 mg/L ampicillin). Cultures were incubated at 37° C overnight.

Plasmid Midi Prep and Determination of Insert Orientation

Thirteen single colonies were selected and allowed to grow in LB broth containing 100 µg/ml ampicillin overnight at 37 °C, then transferred to 1.5 ml tubes and centrifuged at 16,000 x G for 5 min. The pellet was resuspended in 0.3 ml of STET Buffer (8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris, pH 8.0). Lysozyme (25 µl at 20 U/ µl) was added, and tubes were placed in boiling water for 60 sec. Samples were then centrifuged at 16,000 x G at room temperature and the pellet removed. Cold isopropanol (300 µl) was added to precipitate the DNA. Samples were centrifuged at 16,000 x G at room temperature and pellets were washed with 70% ethanol. Pellets were then allowed to dry and resuspended in 20 µl T10-E0.1.

To determine insert orientation, a web based tool provided by New England Biolabs (Ipswich, MA) was used to identify restriction sites within the h-VMAT-1
sequence ([http://tools.neb.com/NEBcutter2/index.php](http://tools.neb.com/NEBcutter2/index.php)). Isolated DNA was digested with Xmn1 (New England BioLabs Ipswich, MA). The plasmid was digested with 0.5 µl Xmn1 (20U/µl) in 0.5 µl 10X enzyme buffer (New England Biolabs) supplemented with 0.5 BSA (100µg/ml) and 16 µl ultrapure water. The reaction was allowed to incubate for one hour. Reaction product was subjected to gel electrophoresis to visualize resultant bands conferring proper insert orientation. Clones that contained the correct insert orientation presented bands similar in size at 5563 bp and 4145 bp (Figure 3). Products were confirmed by gel electrophoresis in five of thirteen samples. These plasmids were selected for site directed mutagenesis and sequencing.
Figure 3. **XMN-1 Digestion of hVMAT1-pCMV6-Neo to Determine Insert Orientation.** Human VMAT1 insert was removed from the original vector with the Not-1 restriction enzyme. The insert was then ligated into the pCMV6-Neo vector with the T4 ligase. Orientation of the insert was determined by digestion of the plasmid with XMN-1 restriction enzyme. Correct orientation yielded bands of 5563 bp and 4145 bp, whereas, incorrect orientation of the insert yielded bands of 7383 and 2325 bp when analyzed by gel electrophoresis.

**Site Directed Mutagenesis of hVMAT1 in Neomycin Vector**

In order to create the nucleotide polymorphism of interest, site directed mutagenesis was performed with the QuickChange II XL Site Directed Mutagenesis Kit ( #200521, Stratagene La Jolla, CA) according to manufacturer’s protocol. Primers for mutagenesis were determined with the QuickChange Primer Design Program (http://www.stratagene.com/sdmdesigner/default.aspx) and purchased from (Invitrogen). Primers spanned the target area and are represented in Table 2.
**Table 2. Mutagenesis and Sequencing Primers**

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C277A</td>
<td>GGCCATCACCATGCTCCGGACCATTCTG</td>
<td>CCGGTAGTGTACGAGGCGCCTGGTAAG</td>
</tr>
<tr>
<td></td>
<td>GGACTTTCCAAATGTGC</td>
<td>ATTAGGACAAGGCTGGTG</td>
</tr>
<tr>
<td></td>
<td>CCTGACCTCCCTGTGTTTCC</td>
<td>GCCCGGACCTGGGGGACGTCTTCCC</td>
</tr>
<tr>
<td></td>
<td>CCCTTACATCCTGTTGCTGC</td>
<td>CAGACGAAAGGAAAACCTCAC</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGTGGCTGTGTCCCTTAATC</td>
<td>CCCAAAGGCCAGGCCCC</td>
</tr>
<tr>
<td></td>
<td>GCCCTCGCTCTCTCTGCC</td>
<td>CCTCCCCCAGAGGAAATTCC</td>
</tr>
<tr>
<td></td>
<td>CGGAAGAGTGTTACC</td>
<td>GAGGTAACATCTTTCCG</td>
</tr>
<tr>
<td></td>
<td>GGCCCGAACCCCTCAAGGC</td>
<td>CCTCCCCCAGAGGAAATTCC</td>
</tr>
</tbody>
</table>

**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% CO₂ in DMEM medium (# 30-2002, ATCC) containing 4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate. COS-1 medium is supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin.

CHO K1 cells were obtained from Dr. Bruno Steiger, Geneva Switzerland and maintained at 37°C with 5% CO₂ in DMEM medium (#11885-092, Invitrogen) containing 4 mM L-glutamine, 5 mM glucose and 0.4 mM glycine. Medium was supplemented with 10% heat inactivated fetal bovine serum, 50 μg/ml L-proline, 100 units/ml penicillin and 100 units/ml streptomycin. Because CHO cells were transfected with plasmids containing a gene that confers neomycin resistance, these cells were maintained in a medium...
supplemented with 500 μg/ml Geneticin (# 10131-035, Invitrogen). As the cells grow and divide, the medium selects against cells that do not have the plasmid.

Cell Transfection

Cells were transfected with VMAT-1 cDNA or vector alone by electroporation as described by Finn et. al. COS-1 cells were grown to confluency in T-75 flasks and fed fresh medium 24 hours prior to electroporation. The day of electroporation, cells were rinsed with calcium-and magnesium-free phosphate buffered saline (PBS), detached with 0.25% trypsin and medium containing serum added to inactivate trypsin. Cells were pelleted by centrifugation (500 x g for 5 min at 4°C). COS 1 cells were resuspended in 700 μl of warm PBS containing calcium and magnesium (Invitrogen), whereas CHO cells were resuspended in ice-cold PBS containing calcium and magnesium (Invitrogen) and remained on ice for 10 min prior to electroporation. The cell suspension and 15 μg of DNA was transferred to a 0.4 cm gap cuvette in the Gene Pulser II (Bio-Rad, Hercules, CA). Suspension and DNA were electroporated at 0.4 kV and 950 μF. CHO cells were placed on ice for 10 min prior to replating. Cells were replated in a T-75 flask containing fresh medium and incubated prior to membrane preparation for transport assays.

Membrane Preparation

The third day after electroporation, cells were rinsed with PBS. After removal of PBS, lysis buffer (containing 0.32 M sucrose, 10 mM Hepes adjusted to pH 7.4 with 1M
KOH) supplemented with proteolytic inhibitors as described by Finn et. al (7), was added to the T-75 flask. Cells were then scraped with a cell scraper into the lysis buffer. The cell suspension was pelleted (500 x g for 5 min) and the supernatant removed. Cells were then resuspended in 100 μl lysis buffer. The cell suspension was homogenized 40 strokes with a Dounce homogenizer, and homogenate was pelleted at 3500 x g for 10 min at 5 °C. The supernatant was taken and aliquotted, and the pellets were resuspended in 150 μl of SH buffer with proteolytic inhibitors and aliquotted. Total protein for the supernatant was measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Aliquots were stored at -70 °C until assayed for VMAT-1 activity.

**Western Blotting**

CHO-K1 and COS-1 cells membranes were prepared as described above. With the help of Dr. Krista Stenger (University of Richmond, Richmond, VA), proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated overnight at 4 °C or for 1 hour at room temperature in blocking solution containing Tris buffered saline, 0.1% Tween-20 (TBST) and 5% dry milk, and subsequently incubated overnight at 4 °C with a primary goat polyclonal anti-VMAT-1 antibody (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-7718) diluted 1:200 in TBST. This antibody targets the COOH-terminal end of human VMAT1. Blots were washed with TBST and incubated with a 1:7500 dilution of bovine anti-goat IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., sc-2378) for 1 hour at
room temperature. The blots were visualized with enhanced chemiluminescence (Amersham, Biosciences, UK). Blots were stripped using Re-Blot Plus Mild for 15 min, blocked 2X for 5 min with blocking solution and probed again with a mouse monoclonal anti-actin antibody (clone AC-15; Sigma) diluted 1:5000 in TBST for 1 hour at room temperature. Blots were washed with TBST and incubated with a 1:7500 dilution of goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., sc-2031) for 1 hour at room temperature and visualized as described above.

A blocking peptide was used to neutralize the reactivity with AB #1: A five-fold excess of peptide was incubated with the appropriate dilution of antibody in 500 μl of TBST overnight at 4 ºC with agitation. Following neutralization the antibody/peptide mixture was added to the appropriate final working volume of TBST as a substitute for the primary antibody alone, and the western blot procedure was performed as usual.

VMAT Uptake Assay

Each assay contained 30 μg of cell protein, 5 mM ATP and 20 nM ³H labeled serotonin [³H-5HT] (Amersham Biosciences, Piscataway, NJ) in a final volume of 200 μl uptake buffer. Tubes were then incubated at 29 °C for 10 min, except in preliminary time course experiments. The reaction was terminated with 1.5 ml cold SH buffer and filtered rapidly through 0.2 μm Supor 200 membrane filters (Pall/Gellman #60300) in a Millipore sampling manifold. The reaction tubes were rinsed with an additional 1.5 ml cold SH buffer and dispensed to the proper filter. Filters were transferred to a scintillation vial
containing 5 ml of ScintiSafe scintillation fluid (Fisher Scientific, Atlanta, GA) and reactivity determined with a Beckman LS6000IC scintillation counter. The reaction kinetics were analyzed with Prism software (GraphPad Prism, La Jolla, CA).

Statistics

Statistical analysis was performed using the GraphPad Prizm4 program (GraphPad Software Inc, La Jolla, CA).

Results

Site Directed Mutagenesis of hVMAT1-pCMV6-Neo

A BLAST alignment with a GenBank reference sequence for human VMAT-1 (GenBank # NM_003053.1) revealed that the Origene human VMAT1 sequence contained the A277C SNP of interest. The SNP results a cytosine (c) to adenine (a) change resulting in a threonine rather than a proline at residue 4 of the protein. To determine if this finding was correct, a sample of the Origene human VMAT1 was sequenced by the Sequencing Core at VCU (Figure 4A). Indeed, the A277C SNP was found to be present in the human DNA provided by Origene (Rockville, MD SC122643). Site directed mutagenesis to revert the C to A, and the mutagenesis was verified to be successful and reflected wild type human VMAT1 (VMAT, Figure 4B). Both VMAT1 and A277C were fully sequenced (Figures 5 and 6).
Creation of Stably Transfected Cell Lines

Western blot analysis confirmed the presence of hVMAT1 protein in transfected cell extracts (Figures 7 and 8). Three immunoreactive bands at 55, 58 and 72 kDa respectively were detected in the pellet fraction of CHO wild type cells. This indicates the presence of cross-reactivity of hamster VMAT1 with the antibody targeting the human VMAT1 C-terminus. In cells transfected with human VMAT1 carrying the A277C mutation, the 55 kDa band was either absent (Figure 7) or reduced in the protein pellet fraction (Figure 8). The 58 kDa band was more evident, however, in cells with the A277C mutation than in non-transfected wild type cells, suggesting the transfection was successful. Cells transfected with human VMAT1 matching the reference sequence (GenBank: #NM_003053.1; indicated as VMAT in Figures 7 and 8) exhibited over-expression of the 58 kDa band and slightly increased expression of the 55 kDa band over that observed in wild type CHO cells.

Results of densitometric analysis of the 55 and 58 kDa bands are shown in Figure 9. A277C pellet produced a 58 kDa band 0.5 times the density of its respective actin band. A277C pellet did not produce a measurable 55 kDa band. VMAT1 protein pellet produced bands at 55 kDa and 58 kDa, which showed a density of 1.6 and 0.8 times their respective actin bands. VMAT1 supernatant produced one band at 58 kDa which measured 0.8 times the density of the actin band. WT CHO supernatant did not produce measurable band at 55 or 58 kDa. WT pellet however, produced bands at 55 and 58 kDa. These bands measured 0.36 and 0.3 times the respective actin bands.
**Analysis of Serotonin Binding Activity**

Assay of serotonin binding activity of protein from permanently transfected and wild-type CHO cells or transiently transfected and wild type COS1 cells was analyzed by radioactive ligand binding assay. Binding activity of either supernatant or pellet protein fractions from transfected CHO and COS1 cells showed no statistical difference in activity when compared to protein from wild-type cells (Figures 10 and 11).

**Discussion**

The A277C mutation has been linked to schizophrenia in more than one nationality by several laboratories. The effect of this mutation on the function and expression of VMAT1 is unknown. This study was one of the first to investigate the effect of SNPs in the human VMAT1 gene on cellular expression of the VMAT1 protein. DNA was obtained from a manufacturer and found to contain the A277C SNP of interest. Site directed mutagenesis was successfully performed, resulting in a sequence matching the reference sequence (GenBank: #NM_003053.1) for use in stable transfections.

Transfection of human VMAT1 into CHO cells was successful as determined by Western blot analysis. Western blotting also revealed several points of interest. In cells
with the A277C SNP, immunoreactive VMAT1 proteins (58 and 72 kDa) were detected, but expression of the expected protein band at 55 kDa was either reduced or absent. This does not appear to be due to failure of the antibody to bind to the mutated region of the sequence, as the A277C mutation is near the NH2 terminus of the protein, and the antibody targets the COOH terminus. Alternatively, lack of the 55 KDa VMAT1 may reflect other effects on VMAT1 processing that made the protein more vulnerable to degradation. This hypothesis can be explored by immunoprecipitation and subsequent western analysis for ubiquitinated VMAT1.

Binding assays revealed no difference in activity between any cell treatment: WT, A277C or VMAT1. Both pellet and supernatant were tested for the activity of VMAT1. Wild type cells showed relatively high binding of tritiated serotonin. The Western analysis revealed the presence of immunoreactive hamster VMAT1 in the pellet fraction of wild-type cells, accounting for the high binding. However, use of positive controls did not yield results observed in the past. This confirms that the assay procedure may need to be revised. Also, we cannot exclude the possibility that wild type or mutated human VMAT1 that is expressed in CHO cells may have very little activity. This hypothesis cannot be confirmed without further study.

This study has provided a preliminary base for performing future experiments. Additional transport assays would provide great insight into the effects of mutation on activity of human VMAT1. Despite extensive troubleshooting of the transport assay, measurable VMAT1 activity was not seen. The assay protocol may have to be revised. A
different transport assay utilizing intact cells may provide a better measure of VMAT1 activity in transfected cells.

Further Western blot analysis utilizing various molecular weight ladders and antibodies are required to obtain a clearer picture of the expression of VMAT1 within transfected cells. The amount of ubiquitinated VMAT1 within A277C samples could be tested in order to determine whether the A277C VMAT1 is in fact degraded quickly after formation. In order to achieve this goal, proteasome activity in cells would have to be inhibited.

This study provides the first evidence of the affect of the A277C SNP on the formation of the VMAT1 protein in mammalian cells. Stably transfected cell lines containing either VMAT1 or A277C have been successfully created. It was determined that the A277C SNP results in a protein change of VMAT1 eliminating the 55 kDa product as determined by western blot analysis. This novel study provides a great base for future study of the A277C mutation.
Figure 4. Site Directed Mutagenesis of VMAT 1 sequences. (A) Sequencing of the VMAT1 DNA provided by Origene (Rockville, MD) indicated the presence of SNP A277C highlighted in pink. The sequence is aligned with the hVMAT1 reference sequence (GenBank NM_003053.1). (B) The Origene DNA was subjected to site directed mutagenesis, which reverse-mutated A277C to a VMAT sequence matching the hVMAT1 reference sequence (GenBank: # NM_003053.1) as highlighted in yellow.
After obtaining the plasmid, it was sent to the sequencing core (VCU). It was determined that the plasmid contained the SNP of interest and the remainder of the sequence was identical to human VMAT1 (Gen Bank: #NM_003053.1). Start and stop codons are highlighted.
Figure 6. Sequence of hVMAT1 DNA Subject to Site Directed Mutagenesis (VMAT). After site directed mutagenesis, the plasmid was sent to the sequencing core (VCU). It was determined that the plasmid was mutated successfully to match the reference sequence and the remainder of the sequence was identical human VMAT1 (GenBank: #NM_003053.1). Start and stop codons are highlighted.
Figure 7. Expression of VMAT1 Protein in CHO-K1 Transfected Cells. Stably transfected CHO-K1 cell lines containing either the A277C mutation (Origene sequence) or the reference VMAT1 sequence created with site-directed mutagenesis. S1 denotes cell lysate supernatant, and P1 denotes the 3500 x G cell lysate pellet. WT denotes wild type CHO cell protein (cells not transfected).
Figure 8. Expression of VMAT1 Protein in CHO-K1 Transfected Cells. (S1 = supernatant and P1 = pellet) Stably transfected CHO-K1 cell lines expressed either the A277C mutation (Origene sequence) or the wild type VMAT1 sequence created with site-directed mutagenesis. S1 denotes cell lysate supernatant. P1 denotes cell lysate pellet. WT denoted wild type CHO protein. The above blot utilized cell protein from a separate harvest from protein in Figure 7.
Figure 9. Expression of hVMAT1 in Transfected Cells. CHO cells were transfected with either the A277C or VMAT1 plasmids and harvested when confluent. Cell lysate supernatant and pellet were subject to western blot analysis by Dr. Krista Stenger (University of Richmond). Density of resultant bands were measured with ImageJ (NIH) and compared to their respective β-actin bands.
**Figure 10. Binding of Serotonin to Transfected hVMAT1 Protein.** CHO-K1 cells were stably transfected with either the A277C or VMAT1 plasmids. Cell lysate pellet (P1) protein was incubated for 10 min in the presence of 5mM ATP and 20 nM tritiated serotonin.
Figure 11. Serotonin binding activity in transiently transfected COS-1 Cells. COS1 cells were transiently transfected with either the A277C or VMAT1 plasmids. Cell lysate pellet and supernatant protein activities were analyzed by radioactive ligand binding assay. Cell supernatant and pellet demonstrated significant differences in activity despite cell treatment (* p > 0.05 compared to all S1 fractions). No significant differences were observed in activity among wt and transfected cells.
Reference List


VITA

Abena B. Watson- Siriboe was born on January 20, 1985 in Montclair, California and is an American citizen. She graduated from Canyon High School in Anaheim Hills, CA in 2001. Abena received her Bachelor of Science in Biological Sciences and Minor in Neuroscience from University of California, Riverside in 2006. She volunteered in a Neuroscience lab before joining the Master of Science in Biology program at Virginia Commonwealth University, Richmond, Virginia.