Vesicular monoamine transporter-1 (VMAT-1) mRNA and immunoreactive proteins in mouse brain

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Abstract

OBJECTIVE: Vesicular monoamine transporter 1 (VMAT-1) mRNA and protein were examined (1) to determine whether adult mouse brain expresses full-length VMAT-1 mRNA that can be translated to functional transporter protein and (2) to compare immunoreactive VMAT-1 proteins in brain and adrenal.

METHODS: VMAT-1 mRNA was detected in mouse brain with RT-PCR. The cDNA was sequenced, cloned into an expression vector, transfected into COS-1 cells, and cell protein was assayed for VMAT-1 activity. Immunoreactive proteins were examined on western blots probed with four different antibodies to VMAT-1.

RESULTS: Sequencing confirmed identity of the entire coding sequences of VMAT-1 cDNA from mouse medulla oblongata/pons and adrenal to a Gen-Bank reference sequence. Transfection of the brain cDNA into COS-1 cells resulted in transporter activity that was blocked by the VMAT inhibitor reserpine and a proton ionophore, but not by tetrabenazine, which has a high affinity for VMAT-2. Antibodies to either the C- or N- terminus of VMAT-1 detected two proteins (73 and 55 kD) in transfected COS-1 cells. The C-terminal antibodies detected both proteins in extracts of mouse medulla/pons, cortex, hypothalamus, and cerebellum but only the 73 kD protein and higher molecular weight immunoreactive proteins in mouse adrenal and rat PC12 cells, which are positive controls for rodent VMAT-1.

CONCLUSIONS: These findings demonstrate that a functional VMAT-1 mRNA coding sequence is expressed in mouse brain and suggest processing of VMAT-1 protein differs in mouse adrenal and brain.

INTRODUCTION

Vesicular monoamine transporters 1 and 2 (VMAT-1 and VMAT-2) permit vesicular uptake, storage, and regulated release of serotonin, catecholamines, and other biogenic amines. Vesicular uptake prevents rapid degradation of monoamines in the cytoplasm, reduces cytoplasmic production of toxic metabolites of dopamine and other monoamines, and sequesters neural toxins, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine) (Guillot & Miller 2009). The VMAT-1 gene (solute carrier family 18 member 1 = scl18a1) initially was cloned from PC12 pheochromocytoma cells.
and the VMAT-2 gene (member 2 = scl18a2) was screened from a rat brainstem cDNA library (Liu et al. 1992). VMAT-1 and VMAT-2 were designated chromaffin granule amine transporter and synaptic vesicle amine transporter, respectively. Localization with in situ hybridization and immunohistochemistry confirmed that VMAT-1 is the major VMAT in rat and mouse adrenal medulla and VMAT-2 is the only VMAT in most areas of human and rodent brain (Erickson et al. 1992; Liu et al. 1992; Erickson et al. 1996; Peter et al. 1994; Weike et al. 1994; Eiden et al. 2003). Nevertheless, VMAT-1 mRNA has been detected in embryonic rat brain (Hansson et al. 1998) rat pineal (Hayashi et al. 1999), and a population of interneurons in mouse striatum (Ibanez-Sandoval et al. 2010).

Bly’s discovery that a human VMAT-1 gene polymorphism is associated with schizophrenia in a Caucasian American population (Bly 2005) stimulated new interest in a potential role for VMAT-1 in brain. Subsequently three other laboratories reported associations of VMAT-1 gene polymorphisms with neuropsychiatric disorders in European-American, Japanese and Chinese populations (Lohhoff et al. 2006; Richards et al. 2006; Chen et al. 2007). Additionally, Lohhoff et al detected VMAT-1 mRNA and immunoreactive protein in several regions of human brain (Lohhoff et al. 2006).

Data are lacking regarding expression of VMAT-1 in regions of mouse brain other than striatum, and it is unclear whether there are differences in VMAT-1 proteins in brain and adrenal chromaffin cells. Detection of human variants of VMAT-1 mRNA predicting shortened isoforms of the VMAT-1 protein (GenBank accession #s NM_001142324, NM_001142325) (Essand et al. 2005) suggest examination of the entire coding sequence of VMAT-1 mRNA is needed to verify that a full length mRNA is present. Furthermore, immunohistochemical evidence against brain expression of VMAT-1 (Eiden et al. 2003; Weike & Eiden 2000) suggests the need to compare immunoreactive VMAT-1 proteins in the brain and adrenal. This study addresses these issues.

**MATERIALS & METHODS**

**Tissues**

Female CBA/J or ICR mice (6–8 weeks of age) were obtained from Harlan (Indianapolis, IN), and CD-1 male and female mice (retired breeders) were obtained from Charles River (Wilmington, MA). All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Mice were euthanized with CO2, and adrenals and brains were removed, dissected, frozen on dry ice, and stored at –70° C until extracted for RNA or protein.

**Cells**

The COS-1 kidney fibroblast and PC12 pheochromocytoma cell lines were obtained from American Type Culture Collection and maintained at 37°C in a 5% CO2 atmosphere. COS-1 cells were incubated in ATCC’s DMEM (ATCC # 30-2002) containing 4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate and supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. PC12 cells were maintained in DMEM from Invitrogen (1190-044) supplemented with 2 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 10% horse serum, 5% heat inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

RT-PCR was performed as previously described (Andreassi, II et al. 1998) with total RNA extracted with TRI Reagent™ from Molecular Research Center
(Cincinnati, OH) according to procedures provided by the manufacturer. Three μg of RNA were reverse transcribed with the Superscript™ Preamplification System for First Strand cDNA synthesis (Invitrogen, Carlsbad, CA). Negative controls included a reaction without RNA for each assay, and reactions without reverse transcriptase for each sample.

PCR Primers (Table 1) were designed with Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) and purchased from Integrated DNA Technologies (Coralville, IA). All primer pairs spanned at least one intron to permit detection of PCR products resulting from contamination with genomic DNA. Each PCR reaction contained 2 μl of the RT reaction product, 0.4 μM primers and reagents as previously described (Andreassi, II et al. 1998). The cDNA was amplified 38 cycles for the target and 25 cycles for β-actin (15 sec at 94 °C, 15 sec at 56 °C, 30 sec at 72 °C), as amplification was observed to be linear at these cycle numbers. PCR for β-actin was performed on each sample to verify the integrity of the RNA. PCR products were resolved in 1.0% agarose gels stained with ethidium bromide and visualized with the Eagle Eye II Image Analyzer (Stratagene, La Jolla, CA).

Cloning, sequencing, and cellular transfection of VMAT-1 cDNA
Prior to sequencing, PCR amplified cDNA was inserted into the pGEM-T Easy Vector (Promega, Madison, WI) and introduced into E. coli strain DH5α. Three clones positive for the DNA insert were randomly selected for sequencing. Each sample was sequenced with previously described methods (Rudd et al. 2005) in four fragments with VMAT-1D and VMAT-1E forward primers and VMAT-1G and VMAT-1D reverse primers (Table 1).

To examine VMAT-1D expression, the cDNA clone was inserted into an expression vector with SRα promoter (Takebe et al. 1988) and grown in E. coli DH5α. COS-1 cells were transfected with the VMAT-1 cDNA or the vector alone by electroporation as described by others (Finn et al. 1998; Peter et al. 1994). The cells were grown to confluence in T-75 flasks (CoStar) and fed grown to confluence in T-75 flasks (CoStar) and fed of freshly prepared ice cold buffer containing 0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 0.5% Triton-X 100, 2 μg/ml leupeptin, 1 μg/ml aprotinin and 0.2 mM phenylmethylsulfonylfluoride. Lysates were centrifuged at 10,000 x g for 20 min at 4 °C. Protein concentrations of the supernatants were determined with the Bio-Rad assay. Proteins were separated by electrophoresis on either 10% or 12% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated overnight at 4 °C in blocking solution containing Tris buffered saline, 0.1% Tween-20 (TBST) and 3% dry milk, and subsequently incubated either 1 h at room temperature or overnight at 4 °C with one of the following primary antibodies (designated AB) diluted in TBST as indicated in the figures: AB #1 – 1:500 rabbit anti-rat VMAT-1 C-terminus AB1597P from Chemicon; AB #2 – 1:5000 rabbit anti-rat VMAT-1 C-terminus H-V002, Phoenix Pharmaceuticals; AB #3 – 1:500 rabbit anti-rat VMAT-1

Membrane preparation
On the third day after electroporation, COS-1 cells from each T-75 flask were rinsed with PBS, detached, and pelleted as above. The pellet was washed twice in PBS, centrifuged after each wash, and resuspended in 250 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 as recommended by Finn et al. (Finn et al. 1998). The suspension was transferred to a Dounce homogenizer on ice and homogenized with 40 passes, then centrifuged at 20,000 x g for 20 min at 4 °C. The pellet was resuspended in 225 μl of uptake buffer (SH buffer containing 4 mM MgSO4 and 4 mM KCl (without proteolytic inhibitors). The suspension was homogenized as above and total protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots were stored at –70 °C until assayed for VMAT activity.

Uptake assay
Each assay tube contained 100 μg of cell protein, 5 mM ATP, and 20 nM radiolabeled serotonin (3H-5HT) = 5-H[G-3H]T creatine sulfate 15-18 Ci / mmol (Amer sham Biosciences, Piscataway, NJ) in a total volume of 200 μl of uptake buffer. All incubations were conducted at 29 °C for 10 min, except in time course experiments. The reaction was terminated by addition of 1.5 ml cold SH buffer and rapid filtration through 0.2 μm Supor 200 membranes (Pall/Gelman # 60300) in a Millipore sampling manifold. The reaction tubes were washed with an additional 1.5 ml of cold SH buffer, which was transferred to the appropriate filter and rapidly filtered as above. Filters were transferred to 10 ml of Scinti-safe™ scintillation fluid (Fisher Scientific, Fair Lawn, New Jersey), and radioactivity was measured with a Beckman LS6000IC scintillation counter.

Western blotting
Brain regions or cultured cells were lysed in 0.5–1.8 ml of freshly prepared ice cold buffer containing 0.05 M Tris (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 0.5% Triton-X 100, 2 μg/ml leupeptin, 1 μg/ml aprotinin and 0.2 mM phenylmethylsulfonylfluoride. Lysates were centrifuged at 10,000 x g for 20 min at 4 °C. Protein concentrations of the supernatants were determined with the Bio-Rad assay. Proteins were separated by electrophoresis on either 10% or 12% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated overnight at 4 °C in blocking solution containing Tris buffered saline, 0.1% Tween-20 (TBST) and 3% dry milk, and subsequently incubated either 1 h at room temperature or overnight at 4 °C with one of the following primary antibodies (designated AB) diluted in TBST as indicated in the figures: AB #1 – 1:500 rabbit anti-rat VMAT-1 C-terminus AB1597P from Chemicon; AB #2 – 1:5000 rabbit anti-rat VMAT-1 C-terminus H-V002, Phoenix Pharmaceuticals; AB #3 – 1:500 rabbit anti-rat VMAT-1
N-terminus SC-15313, Santa Cruz Biotechnology; AB #4 – 1:200 goat anti-human VMAT-1 C19, Santa Cruz Biotechnology. Blots were washed with TBST and incubated with either 1:5,000 or 1:10,000 dilutions of the appropriate anti-IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, cat #E-1403). The blots were visualized with enhanced chemiluminescence (Amersham, Biosciences, UK).

**Statistical analyses**

Analysis of variance and Tukey’s multiple comparison test were used to evaluate effects of transport inhibitors on uptake of radiolabeled serotonin, and an unpaired t-test was used to compare transport in transfected and wild type COS-1 cells at various time points. Time course of transport was plotted with Prism software (Graphpad, San Diego, CA).

**RESULTS**

**VMAT-1 mRNA in mouse brain**

VMAT-1 mRNA was detected in medulla oblongata and pons (medulla/pons) as well as adrenal of CBA/J female mice. Four sets of PCR primers (Table 1 and Figure 1) verified this finding and did not detect alternative mRNA splice forms. Using primer set D, which permits amplification of the entire translated region of mouse VMAT-1, we also detected VMAT-1 mRNA in the medulla/pons of both male and female CD-1 mice (Figure 1), indicating that the VMAT-1 transcript is expressed in the brains of more than one strain and gender of mice.
Brain VMAT-1 cDNA sequence
Sequencing of PCR products (VMAT-1D) from CBA/J mouse adrenal and medulla resulted in identical 1708 nucleotides. Sequence analyses were repeated, chromatographs were visually inspected to ensure accuracy, and the mouse brain sequence was submitted to GenBank (accession #AY779336). BLAST comparison of the brain cDNA sequence (GenBank accession #NM_153054.2) revealed 99% identity to bases 343 – 2050 of mouse VMAT-1 NM_153054.2. Four nucleotide differences that were observed at positions 540, 717, 1422 and 1746 (numbered according to the reference sequence) were within the coding region (nucleotides 466-2031) but did not result in differences in the predicted amino acid sequence of VMAT-1.

Brain VMAT-1 cDNA expression in COS-1 cells
Transfection of cloned VMAT-1D cDNA into COS-1 cells increased VMAT-1 transport activity approximately 15-fold as assayed by uptake of radiolabeled serotonin (Figure 2). As expected for VMATs, transport activity was inhibited by reserpine and the proton ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Transport was not inhibited by tetrabenazine (Figure 2), which is a high-affinity inhibitor of VMAT-2 activity. These findings indicated that VMAT-1 mRNA in the mouse medulla/pons codes for a functional VMAT-1 protein when expressed in COS-1 cells.

Immunoreactive VMAT-1 in mouse brain detected by western blotting
Western blotting with commercial antibodies to rodent VMAT-1 (AB #s 1, 2, and 3) and human VMAT-1 (AB #4) indicated a common 73 Kd protein in mouse adrenal, brain, and PC12 cells (Figure 3, panels 1, 2, 3, 6). AB #2 (Figure 3, panel 2) that is most commonly used for immunohistochemistry and AB #4 (Figure 3, panel 6) only weakly detected the 73 Kd protein in brain. Higher molecular weight immunoreactive proteins also were detected by several antibodies in PC12 cells and in adrenal but not in brain, and an 80 Kd protein was weakly detected in medulla oblongata by only the N-terminus antibody (Figure 3, panel 3). AB #1 to the C-terminus antibody strongly detected both a 73 and 55 Kd protein in COS-1 cells transfected with brain VMAT-1 cDNA but not in electroporated wild type COS-1 cells (Figure 3, panels 4 and 5). The anti-C-terminus antibodies AB #1 (Figure 3, panels 1, 5, and 7) and AB #4 (Figure 3, panel 6) strongly detected the 55 Kd protein in mouse brain samples but not in adrenal. The C-terminus antibody commonly used for immunohistochemistry (AB #2) never detected the 55 Kd protein (Figure 3, panel 2), whereas the anti-N-terminus antibody weakly detected this protein in mouse medulla oblongata and adrenal (Figure 3, panel 3). Surprisingly, this protein appeared to be almost as abundant in cerebellum, which has little monoaminergic innervation (Efange 2000) as in other brain areas (Figure 3, panels 6 and 7).

DICUSSION
This study provides evidence for VMAT-1 mRNA and immunoreactive protein in the brains of adult mice. No alternative splice forms of the mRNA were detected with several sets of PCR primers. Production of robust VMAT-1 activity in COS-1 cells transfected with the brain VMAT-1 cDNA demonstrated that the brain mRNA is potentially functional, but it remains unclear whether VMAT-1 proteins produced in brain are active.

Detection of both 73 and 55 Kd immunoreactive proteins in cells transfected with the brain VMAT-1 cDNA suggested both proteins are derived from the

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**Fig. 3.** Immunoreactive VMAT-1 assayed by western blotting. Beta-actin loading controls are shown below each numbered panel. Total protein (40 μg per lane) was separated by SDS-PAGE on 10 % or 12 % gels. Panels 1, 2, 3: mouse adrenal (AD), medulla oblongata/pons (MD) and rat pheochromocytoma cells (PC12) probed with AB #s 1, 2, 3, respectively. Panel 4 probed with AB #2 and Panel 5 probed with AB #1: mouse medulla/pons (MD), adrenal (AD), C = COS-1 cells electroporated with the vector alone, or C+V1 = COS-1 cells transfected with brain VMAT-1 cDNA. Panel 6 probed with AB #4 and Panel 7 probed with AB #1: mouse adrenal, CX = frontal cortex, HY = hypothalamus, MD = medulla/pons, CR = cerebellum.
brain transcript; however, only the 73 Kd or higher molecular weight proteins were present in adrenal. Although the sequence of brain and adrenal VMAT-1 cDNA predicts a 56 Kd VMAT-1 protein, modifications of the protein by glycosylation and phosphorylation (Eiden et al. 2003; Henry et al. 1994; Yelin et al. 1998) may account for the observed higher molecular weight proteins. Additional investigation is needed to determine whether modification is necessary for functional transport activity of mouse VMAT-1.

It is notable the N-terminus antibody used in these studies detected the 55 Kd protein in COS-1 cells transfected with the brain VMAT-1 cDNA but failed to detect this protein in brain, whereas two C-terminus antibodies clearly detected it. This suggests that the N-terminus of the 55 Kd VMAT-1 may be altered in mouse brain. Furthermore, robust expression of the 55 Kd protein in cerebellum, which has little monoaminergic innervation (Efange 2000), raises questions regarding the function of the 55 Kd protein as a monoamine transporter. Further investigation is required to localize VMAT-1 immunoreactive proteins to specific cell types and to determine functions of these proteins in the mouse brain. A working hypothesis is that the 73 Kd VMAT-1 detected in both brain and adrenal is a functional transporter with the low abundance in brain as indicated by AB #s 2, 3, and 4. It is conceivable, however, that AB#1 strongly detects a modified form of this protein not detected by the other three antibodies.

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