

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

(Erickson *et al.* 1992), 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; Weihe *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 (Lohoff *et al.* 2006; Richards *et al.* 2006; Chen *et al.* 2007). Additionally, Lohoff *et al.* detected VMAT-1 mRNA and immunoreactive protein in several regions of human brain (Lohoff *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; Weihe & Eiden 2000) sug-

gests 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

Tab. 1. PCR and sequencing primers.

Primer Target	Forward Primer 5'-3' corresponding nucleotides	Reverse Primer 5'-3' corresponding nucleotides	Product size (bp)	Accession#
β-actin	AAGGTGTGATGGTGGGAATGG 44-64	GGCGTGAGGGAGAGCATAG 426-444	401	M12481
VMAT-1 A	GGCTTTGATGCAACTTCTGG 894-913	CATGGCTCTCCTCTCTCGT 1124-1143	250	NM_153054
VMAT-1 B	CAATCATCAGGAGCCCACTT 84-103	AGAAAGGGCAGTCTGTCTGG 332-351	268	NM_153054
VMAT-1 C	ATGGGAGTGTCTACGCCATC 1733-1752	ACACTGAGATTCACGCCTCAT 2072-2092	360	NM_153054
VMAT-1 D	CCAGACGAGCTGTAAGTGAGC 302-322	ACACTGAGATTCACGCCTCAT 2072-2092	1790	NM_153054
VMAT-1 E	ACGAGAGAGGAAGAGCCATG 1124-1143 (sequencing only)	--	--	NM_153054
VMAT-1 G		GATGGCGTAGACTCCCAT 1733-1752 (sequencing only)	--	NM_153054

(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 DH5 α . 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 SR α promoter (Takebe *et al.* 1988) and grown in *E. coli* DH5 α . 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 with freshly prepared medium 24 h before electroporation. Prior to electroporation the cells were rinsed with calcium/magnesium-free phosphate-buffered saline (PBS), detached with 0.25% trypsin and 0.03% EDTA, returned to medium containing serum to inactivate the trypsin, and pelleted by centrifugation at 500 \times g for 10 min at 4°C. Cells were resuspended in 800 μl of prewarmed 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 (Bio-Rad, Hercules, CA) and electroporated at 0.4 kV and 975 μF . Cells were again plated in T-75 flasks and incubated for three days prior to preparation of membranes for transport assays or lysis for western analysis.

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 \times 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 (Amersham 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 Scintisafe™ 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 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin and 0.2 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 \times 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

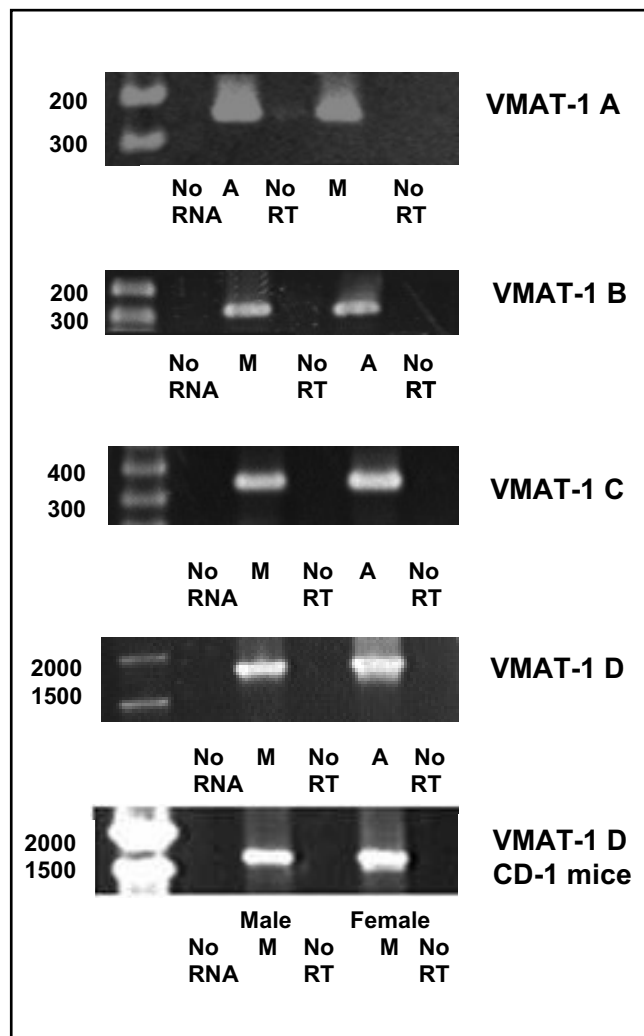


Fig. 1. Expression of VMAT-1 mRNA in medulla/pons and mouse adrenal. Ethidium-bromide stained gels (1.0% agarose) are shown. Base pairs (bp) of the DNA size markers are shown at the left. No RT = samples lacking reverse transcriptase. RT-PCR products include: M = mouse medulla/pons, A = mouse adrenal medulla, No RNA = negative controls for each assay. Three μ g of total RNA were reverse transcribed and 2 μ l of the RT reaction were amplified 38 cycles with primers VMAT-1A -, B -, -C-, -D. Expected sizes of the products A, B, C, D are 250, 269, 360, 1790 bp, respectively. Brain samples were from CBA/J female mice with the exception of the bottom panel with medulla/pons from CD-1 male or female mice.

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

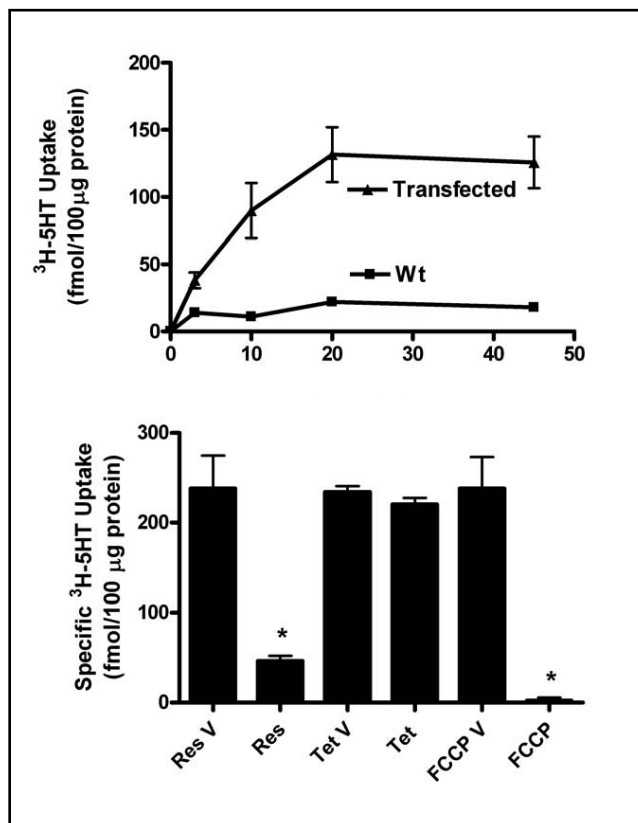


Fig. 2. VMAT-1 transport activity in COS-1 cells expressing mouse brain VMAT-1 cDNA. Membrane protein (100 μ g) from either wild-type cells or cells transfected with the VMAT-1 cDNA was incubated for the times shown or for 10 min in uptake buffer with 5 mM ATP, and 20 nM radiolabeled serotonin (3 H-5HT). Protein from transfected cells was incubated with inhibitors or vehicle for 5 min prior to incubation with 3 H-5HT. Res = 0.1 μ M reserpine, Tet = 5 μ M tetrabenazine, FCCP = 5 μ M carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and V = the vehicle for each inhibitor. * $p < 0.001$

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 and AB #3 to the N-terminus 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 (Efang 2000) as in other brain areas (Figure 3, panels 6 and 7).

DISCUSSION

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-1cDNA 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

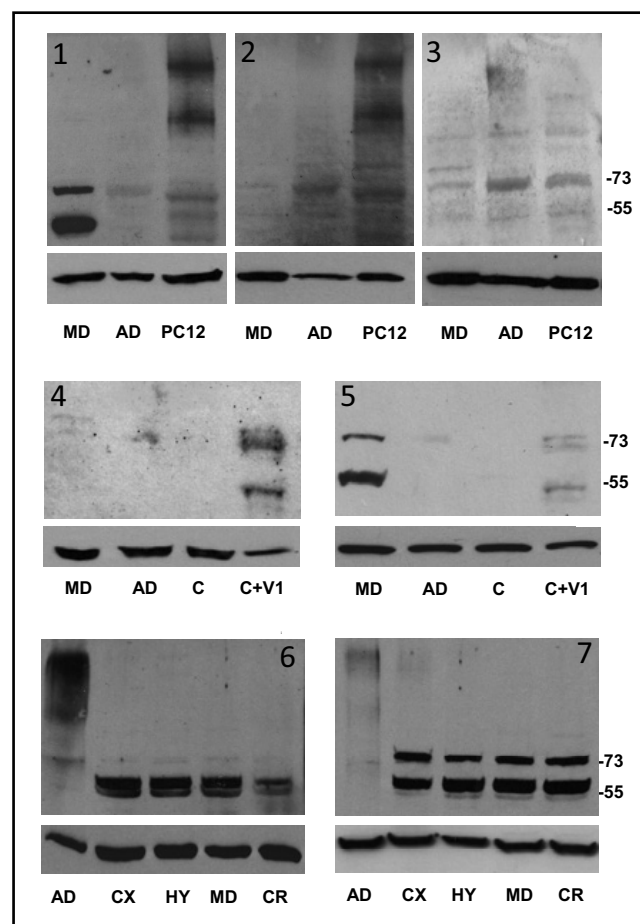


Fig. 3. Immunoreactive VMAT-1 assayed by western blotting. Beta-actin loading controls are shown below each numbered panel. Total protein (40 μ g per lane) was separated by SDS-PAGE on 10 % or 12 % gels. Panels 1, 2, 3: mouse adrenal (AD), medulla oblongata/pons (MD) and rat pheochromocytoma cells (PC12) 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 = COS1 cells transfected with brain VMAT-1 cDNA. Panel 6 probed with AB #4 and Panel 7 probed with AB #1: AD = 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 that 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 (Efang 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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