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# N-TERMINUS REGULATION OF VMAT2 MEDIATES METHAMPHETAMINE-STIMULATED EFFLUX ☆

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**Abstract—The 20 amino acid (AA) N-terminus of the vesicular monoamine transporter 2 (VMAT2) was examined as a regulator of VMAT2 function. Removal of the first 16 or 19 AAs of the N-terminus resulted in a molecule with reduced ability to sequester [$^3$H]-5HT. A glutathione-S-transferase-construct of the N-terminus underwent phosphorylation in the presence of PKC at serines 15 and 18. These putative phosphorylation sites were examined for effects on function. Phospho-mimetic substitution of serines 15 and 18 with aspartate in the full-length VMAT2 resulted in reduced [$^3$H]-5HT sequestration and reduced methamphetamine (METH)-stimulated efflux of preloaded [$^3$H]-5HT. In contrast, mutation of serines 15 and 18 to alanines maintained intact net substrate sequestration but eliminated METH-stimulated efflux of pre-accumulated [$^3$H]-5HT. In summary, these data suggest a model in which the VMAT2 N-terminus regulates monoamine sequestration.** © **2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: vesicular monoamine transporter 2, methamphetamine, kinase, efflux, transporter, monoamine.**

## INTRODUCTION

Among the monoamine neurotransmitter transporters, recent investigations have demonstrated a contribution of the N-terminus to the function of the monoamine plasma membrane transporters (PMTs). In response to amphetamine (AMPH) or methamphetamine (METH) treatment, monoamine PMTs enter an efflux-permissive state, releasing cellular monoamine into the extracellular space. This process was demonstrated to involve phosphorylation of the PMT N-termini; for example, in response to AMPH, protein kinase C (PKC) $\beta_{II}$, a $Ca^{++}$-activated PKC-isotype, caused phosphorylation of the dopamine transporter (DAT) N-terminus in human embryonic kidney-293 cells (Khoshbouei et al., 2004; Cervinski et al., 2005; Johnson et al., 2005; Seidel et al., 2005; Fog et al., 2006; Sucic et al., 2010).

The vesicular monoamine transporter 2 (VMAT2) is responsible for sequestering monoamines from the cytosol of monoaminergic cells into vesicular compartments for subsequent exocytotic release (Erickson et al., 1992; Liu et al., 1992a,b). Mice completely lacking VMAT2 die a few days after birth (Fon et al., 1997) whereas hypomorphic mice, expressing severely lowered VMAT2, demonstrate Parkinson's disease (PD) symptoms and pathology later in life (Mooslehner et al., 2001; Caudle et al., 2007). A mutation was identified in the VMAT2 amino acid (AA) coding region that severely reduced monoamine transport and correlated with PD symptoms in a Saudi Arabian family (Rilstone et al., 2013). In contrast, VMAT2 gain-of-function promoter haplotypes were shown to correlate with a lower incidence of PD in women (Glatt et al., 2006b). However, despite these findings, evidence correlating polymorphisms in the coding region of the VMAT2 to disease is extremely rare (Glatt et al., 2001, 2006a; Burman et al., 2004).

The VMAT2 is also a target of METH/AMPH drug action and is being investigated as an intervention target for addiction (Zheng et al., 2006; Crooks et al., 2011). Though the molecular details of the process are not well understood, it is the initial throughway for METH/AMPH-triggered efflux of vesicularly-stored monoamines (Pifl et al., 1995; Sulzer et al., 1995, 1996, 2005; Takahashi et al., 1997; Partilla et al., 2006). Additionally, striatal-synaptic VMAT2 expression levels are reduced in rats following METH exposure potentially contributing to METH-induced toxicity by compromising cytosolic DA clearance (Eyerman and Yamamoto, 2007; Fleckenstein et al., 2009).

Unlike the longer PMT N-termini, the hVMAT2 is only 20 AAs in length (Fig. 1). It shares 80% homology with the VMAT1 and similar to the monoamine PMTs the N-terminus is putatively localized to the cytosol

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*Abbreviations:* AAs, amino acids; AMPH, amphetamine; BSA, bovine serum albumin; $CaCl_2$, calcium chloride; CPM, counts per minute; DAT, dopamine transporter; DPM, decays per minute; GST, glutathione-S-transferase; HA, hemagglutinin; kDa, kilodalton; KLH, keyhole limpet hemocyanin; KSR, ketansarin; METH, methamphetamine; $MgCl_2$, magnesium chloride; M-PMTs, monoamine plasma membrane transporters; PBS, phosphate-buffered saline; PD, Parkinson's disease; $PIP_2$, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; pN-term Ab, phospho-specific N-term antibody; PS, diolyl phosphatidyl serine; PVDF, polyvinylidene fluoride; RSP, reserpine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; SH, sucrose HEPES; TBZ, tetrabenazine; TBZ-OH, [$^3$H]-tetrabenazine-OH; VMAT2, vesicular monoamine transporter 2.

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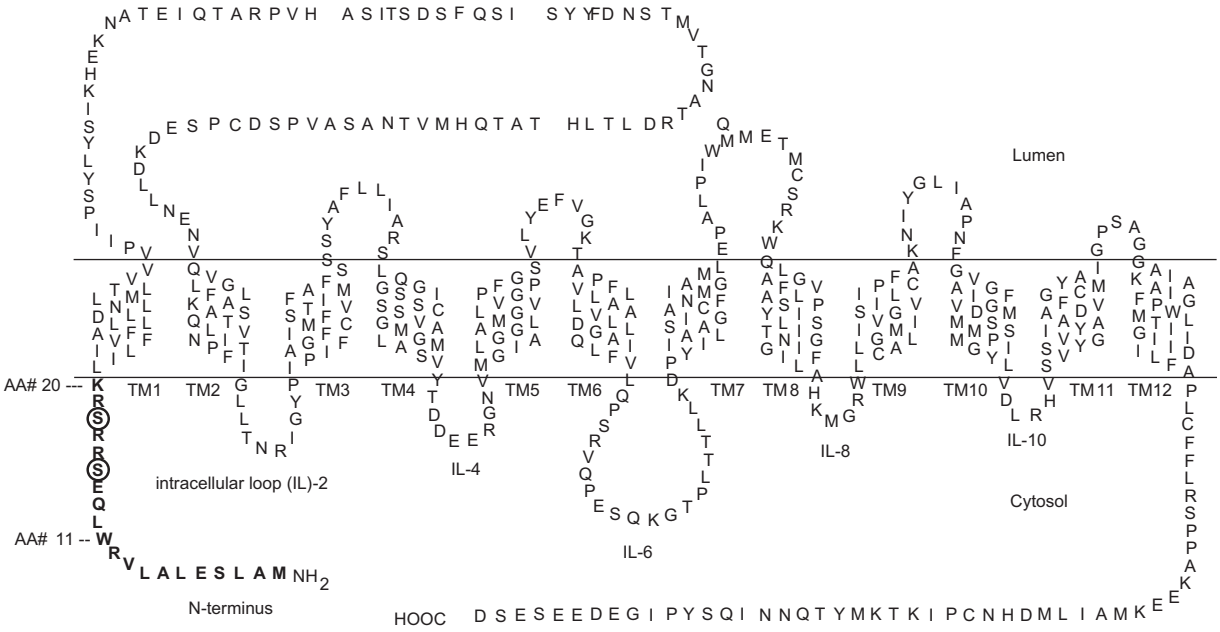


**Fig. 1.** Sequence and structural information for hVMAT2. The 20 AA N-terminus is indicated in bold lettering. Putative PKC phosphorylation sites at serines 15 and 18 (referred to in the text) are circled.

(Erickson et al., 1992; Liu et al., 1992a; Erickson and Eiden, 1993; Howell et al., 1994; Takahashi and Uhl, 1997; Duerr et al., 1999). Previous investigations have ascribed regulatory functions to the VMAT2 C-terminus (Krantz et al., 1997; Tan et al., 1998; Waites et al., 2001; Li et al., 2005) and the large luminal-loop domain between TMs 1 and 2 (Ahnert-Hilger et al., 1998, 2003; Holtje et al., 2000; Brunk et al., 2006; Yao and Hersh, 2007). It had been found that photolabels of the two VMAT2 inhibitors tetrabenazine (TBZ) and ketansarin (KSR) derivatized the N-terminus (Sievert and Ruoho, 1997) indicating a possible regulatory role for the N-terminus. The present study further examined the role of the N-terminus in VMAT2 function and found that the N-terminus regulated the level of substrate-sequestration achieved by the VMAT2 as well as the as VMAT2 efflux-response to METH.

## EXPERIMENTAL PROCEDURES

### Supplies

Cosmic Calf Serum (Hyclone); pGEX vector (Clontech); diolyl phosphatidyl serine (PS; Avanti lipids); PKC (a gift from Paul Bertics PhD, Univ. Wisconsin, Madison); protease inhibitors leupeptin, 4-(2-aminoethyl)a benzenesulfonyl fluoride and phenylmethylsulfonyl fluoride (International Chemical and Nuclear); [$^{32}$P]-γ-ATP (Perkin Elmer); 10,000 kilodalton (kDa) molecular weight cut-off centrifuge filter (Sartorius); polyvinylidene fluoride (PVDF; Millipore); Owl VEP-2 transfer apparatus, 0.1%-Tween casein blocking buffer (ThermoFisher); anti-rabbit secondary antibody (Sigma–Aldrich); horse radish peroxidase (Millipore); the (i) peptides, (ii) the peptide immunogen that was conjugated to keyhole limpet hemocyanin (KLH) in order to generate the phospho-specific N-term antibody (pN-term Ab) and (iii) bovine serum albumin (BSA) derivatized with both phosphorylated and nonphosphorylated forms of the N-terminus were synthesized by Gary Case at the peptide facility, UW-Madison Biotech Center; the pN-term Ab was generated by immunization of rabbits at Cocalico; Sulfolink affinity-column (Pierce); [$^{3}$H]-serotonin (5HT; Perkin Elmer); glass fiber/B filters (Whatman).

### Cell culture, transfections, plasmid constructs

COS-cells were cultured in Dulbecco's modified eagles medium, 10% Cosmic Calf Serum at 5% $CO_2$ at 37 °C. One day prior to transfection, cells were divided to obtain 90–100% confluency on the day of transfection. Cells were trypsinized, washed one time in phosphate-buffered saline (PBS) and resuspended in 500 µl of 'cytomix' (van den Hoff et al., 1992) buffer containing a given amount of DNA. Cell/DNA mixture was transferred to a 4-mm cuvette and electroporated at 950 µF and 226 mV. Cuvettes were placed in a 37 °C 5% $CO_2$ incubator 15 min prior to transferring cells to media to allow the cells to recover from electroporation. Cells were usually used ("harvested") within 24 h of transfection. The WT human VMAT2 construct has been previously described (Thiriot and Ruoho, 2001). Basically the hVMAT2 construct was inserted into pcDNA3.1 (−) at *Xho1* and *HIII* sites. A Flag and a 6-histidine epitope were placed sequentially on the C-terminus. Finally, glycosylation sites were removed as described previously (Thiriot and Ruoho, 2001), and an hemagglutinin (HA) epitope was inserted in the first luminal loop between TMs 1 and 2. The S15/18A and S15/18D N-terminus VMAT2 mutants were created from