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Immunochemical localization of vesicular monoamine transporter 2 (VMAT2) in mouse brain

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ABSTRACT

Vesicular monoamine transporter 2 (VMAT2, *SLC18A2*) is a transmembrane transporter protein that packages dopamine, serotonin, norepinephrine, and histamine into vesicles in preparation for neurotransmitter release from the presynaptic neuron. VMAT2 function and related vesicle dynamics have been linked to susceptibility to oxidative stress, exogenous toxicants, and Parkinson's disease. To address a recent depletion of commonly used antibodies to VMAT2, we generated and characterized a novel rabbit polyclonal antibody generated against a 19 amino acid epitope corresponding to an antigenic sequence within the C-terminal tail of mouse VMAT2. We used genetic models of altered VMAT2 expression to demonstrate that the antibody specifically recognizes VMAT2 and localizes to synaptic vesicles. Furthermore, immunohistochemical labeling using this VMAT2 antibody produces immunoreactivity that is consistent with expected VMAT2 regional distribution. We show the distribution of VMAT2 in monoaminergic brain regions of mouse brain, notably the midbrain, striatum, olfactory tubercle, dopaminergic paraventricular nuclei, tuberomammillary nucleus, raphe nucleus, and locus coeruleus. Normal neurotransmitter vesicle dynamics are critical for proper health and functioning of the nervous system, and this well-characterized VMAT2 antibody will be a useful tool in studying neurodegenerative and neuropsychiatric conditions characterized by vesicular dysfunction.

1. Introduction

The vesicular monoamine transporter 2 (VMAT2, *SLC18A2*) is a twelve-transmembrane glycoprotein within the TEXAN (Toxin EXtruding ANtiporter) family of transporters (Eiden et al., 2004). VMAT2 resides on the membrane of secretory vesicles in monoaminergic neurons of the nervous system and in a variety of secretory cells in the gastrointestinal, endocrine, hematopoietic, and immune systems, and is often co-expressed in the periphery with its non-neuronal isoform, VMAT1 (Anlauf et al., 2006, 2004, 2003; Erickson et al., 1992; Henry et al., 1994; Peter et al., 1995a; Schuldiner et al., 1995; Tillinger et al., 2010; Weihe et al., 1994). VMAT2 utilizes an electrochemical gradient maintained by a vesicular H^+-ATPase to transport one monoamine molecule (dopamine, serotonin, norepinephrine, or histamine) into the highly acidic vesicular lumen in exchange for the efflux of two protons (Chaudhry et al., 2008; Eiden et al., 2004; Erickson et al., 1995; Wimalasena, 2011). The function of VMAT2 is multifold: it prepares neurotransmitters for presynaptic release (Erickson et al., 1992; Henry et al., 1994) and prevents oxidative damage by sequestering deleterious cytosolic monoamines into the vesicle (Alter et al., 2013; Sulzer and Zecca, 2000).

VMAT2 regulates neurotransmitter dynamics and neuronal health (Fon et al., 1997; Wang et al., 1997). Disrupted monoaminergic transmission characterizes a variety of neurodegenerative

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and neuropsychiatric disorders, such as dystonia, Huntington's disease, depression, attention deficit hyperactivity disorder, schizophrenia, and addiction (Creese et al., 1996; Eisenberg et al., 1988; Freis, 1954; Hornykiewicz, 1998; Klawans et al., 1972; Ritz et al., 1988; Song et al., 2012). In the case of Parkinson's Disease (PD), presynaptic monoamine vesicle function is substantially disrupted, and this dysfunction is hypothesized to contribute to neuronal vulnerability in PD pathogenesis (Pifl et al., 2014). *In vitro,* excess cytosolic dopamine causes intracellular damage via formation of reactive oxygen species in cell cultures (Zhang et al., 2000). *In vivo,* our laboratory has shown that a mouse model with 95% decreased VMAT2 expression (VMAT2-LO) has reduced ability to sequester deleterious cytosolic dopamine into vesicles and display a number of age-dependent motor and non-motor symptoms associated with PD (Caudle et al., 2007, 2008; Taylor et al., 2011, 2014). Alternately, our laboratory's mouse model with two-fold VMAT2 protein overexpression (VMAT2-HI) has increased ability to sequester dopamine into vesicles and is protected against dopaminergic degeneration (Lohr et al., 2014, 2015, 2016).

The continuum of VMAT2 gene expression represented by the VMAT2-LO, wildtype (WT), and −HI mice is helpful for confirming the usefulness and specificity of a VMAT2 antibody. Here, we show the use of our polyclonal VMAT2 antibody in a variety of immunochemical assays. The antibody successfully binds to and labels VMAT2, showing specific protein expression in regions that correspond to monoamine production and release (Ciliax et al., 1995; Fujiwara et al., 1999; Mazzoni et al., 1991; Zhou et al., 1996).

Due to stock depletion, the source of a previously-used effective antibody to VMAT2 is no longer available. Though other groups have had success with other commercially available VMAT2 antibodies (Iritani et al., 2010; Shin et al., 2012; Temple et al., 2016; Zhang et al., 2015), we were unsuccessful in using these antibodies to achieve the specificity and selectivity needed for a battery of immunochemical assays. To address this deficit, we have designed a polyclonal rabbit VMAT2 antibody against a peptide in the C-terminal region of mouse VMAT2. Since creating this antibody, our laboratory has received multiple requests for its use, indicating a need for an effective, well-validated VMAT2 antibody. Here, we use our newly-developed antibody to describe the precise cellular and regional distribution of VMAT2 within the mouse brain.

2. Materials and methods

2.1. VMAT2 antibody production

The C-terminal region of mouse VMAT2 (TQNNVQPYPVGDDEESESD) was conjugated to maleimide activated mcKLH (Thermo Scientific) and sent to Bethyl Laboratories (Montgomery, TX, USA) and Covance Custom Immunology Services (Princeton, NJ, USA) to be injected into two rabbits from each company. Initially, animals were immunized with 500 μg conjugated protein per animal and boosted with 250 μg after 2, 4, and 6 weeks and 125 μg every four weeks thereafter. Sera were collected every other week for 6–12 months and sent back to our laboratory. We optimized the immunochemical use of antisera using VMAT2-WT and −LO brains and VMAT2-transfected HEK cells. Bleeds from one rabbit from Covalence Custom Immunology Services yielded polyclonal anti-VMAT2 serum which passed screening in our immunochemical applications.

2.2. Mice

VMAT2-LO mice were generated by backcrossing the original mixed-background VMAT2-deficient strain (Caudle et al., 2008; Taylor et al., 2014) to Charles River C57BL/6 for four generations using a marker-assisted selection (i.e. "speed congenic") approach (Lohr et al., 2016). VMAT2-HI mice were generated as previously described (Lohr et al., 2014). Briefly, we used a bacterial artificial chromosome-mediated transgene to insert three additional copies of the murine *SLC18A2* (VMAT2) gene, including its endogenous promoter and regulatory elements. These founders were then backcrossed to a Charles River C57BL/6 background. Thus, VMAT2-LO, −WT, and −HI mice share the same genetic background. Mice received food and water *ad libitum* on a 12:12 light cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University.

2.3. Differential fractionation

Mouse brains were differentially fractionated using one of three preparations: a crude protein preparation that excludes blood vessels and nuclei, a membrane-associated fraction containing the presynaptic plasma membrane, and the cytosolic vesicle fraction. In all cases, samples were first homogenized in ice-cold homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4) and protease inhibitors (Sigma, 1:1000) using an immersion homogenizer (Tissue Tearor) for approximately 15 s.

Crude protein preparations were achieved by centrifuging samples at 1150 x g for 5 min, then the resulting supernatant was centrifuged at 18400 x *g* for 60 min. The resulting pellet was then resuspended in homogenization buffer.

To produce the membrane-associated fraction and the cytosolic vesicle fraction, sample homogenate was centrifuged at 1000 x *g* for 10 min and the resultant supernatant was centrifuged at 20,000 x g for 20 min. The resultant supernatant was discarded and the pellet was resuspended in homogenization buffer. This resuspended pellet contains isolated synaptosomes. The synaptosomes were osmotically lysed in pure water, then neutralized by addition of HEPES and potassium tartrate (final concentration: 25 mM and 100 mM, respectively). The lysed synaptosomes were centrifuged at 20,000 x *g* for 20 min. The resultant pellet was suspended in assay buffer (25 mM HEPES, 100 mM potassium tartrate, 100 μM EDTA, 50 μM EGA, pH 7.4). This resuspended pellet is the membrane-associated fraction. The supernatant was centrifuged at 120,000 x *g* for 2 h and the resultant pellet was resuspended in assay buffer, producing the cytosolic vesicle fraction. Protein content was determined by BCA assay.

2.4. Immunoprecipitation

Immunoprecipitation was performed using the Pierce co-immunoprecipitation kit (Thermo Scientific) according to manufacturer's protocols. Samples were differentially fractionated into a crude protein preparation, described above. The VMAT2 antibody was cross-linked to agarose beads. Samples were incubated with the antibody-bound columns overnight at 4 °C. Bound protein complexes were eluted the following day and efficacy of immunoprecipitation was determined through immunoblot using the VMAT2 antibody.

2.5. Immunoblot

For the blots in Fig. 1, crude protein preparations from VMAT2-LO, −WT, and −HI striata were prepared as for immunoprecipitation. For the immunoblots shown in Fig. 2, whole brains from VMAT2-WT and −HI animals underwent whole-brain fractionation to yield a membrane-associated fraction and cytosolic vesicle fraction as described above. Samples were *not* boiled. We used 400 mM dithriothrietol (DTT, Sigma) in NuPage LDS Sample Buffer