Methylphenidate Administration Alters Vesicular Monoamine Transporter-2 Function in Cytoplasmic and Membrane-Associated Vesicles

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Abbreviations: DA, dopamine; DAT, dopamine transporter; MPD, methylphenidate; RDE, rotating disk electrode; VMAT-2, vesicular monoamine transporter-2.

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ABSTRACT

In vivo methylphenidate (MPD) administration increases vesicular monoamine transporter-2 (VMAT-2) immunoreactivity, VMAT-2-mediated dopamine (DA) transport, and DA content in a non-membrane-associated (referred to herein as cytoplasmic) vesicular subcellular fraction purified from rat striatum: a phenomenon attributed to a redistribution of VMAT-2-associated vesicles within nerve terminals. In contrast, the present study elucidated the nature of, and the impact of MPD on, VMAT-2-associated vesicles that co-fractionate with synaptosomal membranes after osmotic lysis (referred to herein as membrane-associated vesicles). Results revealed that, in striking contrast to the cytoplasmic vesicles, DA transport velocity vs. substrate concentration curves in the membrane-associated vesicles were sigmoidal, suggesting positive cooperativity with respect to DA transport. Additionally, DA transport into membrane-associated vesicles was greater in total capacity in the presence of high DA concentrations than transport into cytoplasmic vesicles. Of potential therapeutic relevance, MPD increased DA transport into the membrane-associated vesicles despite rapidly decreasing (presumably by redistributing) VMAT-2 immunoreactivity in this fraction. Functional relevance was suggested by findings that MPD treatment increased both the DA content of the membrane-associated vesicle fraction and K⁺-stimulated DA release from striatal suspensions. In summary, the present data demonstrate the existence of a previously uncharacterized pool of membrane-associated VMAT-2-containing vesicles that displays novel transport kinetics, has a large sequestration capacity, and responds to in vivo pharmacological manipulation. These findings provide insights into both the regulation of vesicular DA sequestration and the mechanism of action of MPD, and may have
implications regarding treatment of disorders involving abnormal DA disposition including Parkinson’s disease and substance abuse.
INTRODUCTION

Methylphenidate (MPD) is a commonly prescribed psychostimulant used to treat attention-deficit hyperactivity disorder. It is well established that MPD binds with high affinity to the neuronal dopamine transporter (DAT) where it blocks the inward transport of dopamine (DA) (Wayment et al., 1999; Volz et al., 2005; Volz and Schenk, 2005). In addition, MPD indirectly affects DA transport by the vesicular monoamine transporter-2 (VMAT-2), a transporter protein that is responsible for the sequestration of cytoplasmic DA. Specifically, MPD administration increases $[^3]$H$DA transport into non-membrane-associated (referred to herein as cytoplasmic) vesicles purified from lysates of striatal synaptosomes prepared from treated rats (Sandoval et al., 2002; Sandoval et al., 2003). MPD also increases DA content in the cytoplasmic vesicle subcellular fraction (Sandoval et al., 2002). These phenomena likely result from a redistribution of VMAT-2-containing vesicles within nerve terminals away from membranes and into the cytoplasm (Sandoval et al., 2002).

Recent attention has focused on the regulation of cytoplasmic VMAT-2-containing vesicles after in vivo pharmacological manipulation. In contrast, the present study elucidated the nature of, and the impact of MPD on, VMAT-2-associated vesicles that co-fractionate with synaptosomal membranes after osmotic lysis (referred to herein as membrane-associated vesicles). Results revealed that in striking contrast to cytoplasmic vesicular DA transport, which is characterized by Michaelis-Menten kinetics, DA transport velocity vs. substrate concentration curves in membrane-associated vesicles were of an unexpected sigmoidal shape suggesting positive cooperativity with respect to DA transport (something uncommon in membrane transport proteins and not seen in any
other vesicular neurotransmitter transporter). In addition, transport into membrane-associated vesicles was greater in total capacity in the presence of high DA concentrations than transport into cytoplasmic vesicles. Of potential therapeutic significance, MPD increased DA transport into the membrane-associated vesicles despite rapidly decreasing VMAT-2 immunoreactivity (presumably by redistributing VMAT-2-associated vesicles into the cytoplasm) and thus kinetically upregulated the membrane-associated vesicles. MPD treatment also increased both the DA content of the membrane-associated vesicle fraction and K+-stimulated DA release from striatal suspensions. Taken together, these data suggest the existence of a previously uncharacterized pool of membrane-associated VMAT-2-containing vesicles that display heretofore unreported DA transport kinetics. Of functional relevance, DA transport in this pool, and thus its capacity to affect synaptic transmission, responds to \textit{in vivo} pharmacological manipulation. These findings provide not only novel insights into the physiological regulation of vesicular DA sequestration and synaptic transmission, but also into the mechanism of action of MPD. Accordingly, these data may advance the treatment of disorders involving abnormal DA disposition including substance abuse and Parkinson’s disease.
METHODS

Animals

Male Sprague-Dawley rats (300 - 360 g) were purchased from Charles River Laboratories (Raleigh, NC) and housed in a light- and temperature-controlled room with free access to food and water. All animal procedures were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the University of Utah Institutional Animal Care and Use Committee.

Rotating Disk Electrode (RDE) Measurement of DA Transport Velocities

RDE voltammetry (Schenk et al., 2005; Volz et al., 2006b; Volz et al., 2006a) was used to measure the initial velocities of inwardly directed vesicular DA transport in vesicles purified (Erickson et al., 1990; Teng et al., 1997) from rat striata. Using this technique, DA transport in the clearance profiles is indicated by a downward sloping line as DA is transported into synaptosomes or vesicles where the RDE can not detect it. Each sample consisted of both striata (~60 – 70 mg total wet weight) from a rat that were homogenized in ice-cold sucrose buffer and then centrifuged (800 x g for 12 min at 4 °C) to remove nuclear debris. The resulting supernatant (S1) was centrifuged (22,000 x g for 15 min at 4 °C) to obtain the synaptosomal pellet (P2). The P2 synaptosomal pellet was then resuspended and homogenized in ice-cold water to lyse the synaptosomal membranes. Ice-cold (pH 7.5) 25 mM HEPES and 100 mM potassium tartrate were then added to the synaptosomal pellet homogenate and the resulting mixture was centrifuged (20,000 x g for 20 min at 4 °C) to form the P3 pellet, which contained the membrane-
associated vesicles, and a S3 supernatant. To isolate cytoplasmic vesicles, 1 mM ice-cold (pH 7.5) MgSO₄ was added to the S3 supernatant and the resulting mixture was centrifuged (100,000 x g for 45 min at 4 °C) to obtain the cytoplasmic vesicle pellet (P4). To measure vesicular DA transport, the P3 (membrane-associated vesicles) and P4 (cytoplasmic vesicles) pellets were resuspended in 300 µl of VMAT-2 assay buffer. Some control experiments also monitored synaptosomal (i.e. DAT-mediated) DA transport by resuspending the P2 (synaptosomal) pellet in 300 µl of DAT assay buffer.

The resuspended pellet was placed in a cylindrical glass chamber (10 mm internal diameter with a height of 20 mm) maintained at 37 °C by a VWR International (West Chester, PA) Model 1104 Heating Recirculator and RDE voltammetry was used to measure DA transport as previously described (Volz et al., 2006b; Volz et al., 2006a). A Pine Instruments, Inc. (Grove City, PA) AFMD03GC glassy carbon electrode (5 mm total diameter with a 3 mm diameter glassy carbon electrode shrouded in Teflon) attached to a Pine Instruments MSRX high-precision rotator was lowered into the glass chamber and rotated at 2000 rpm. A Bioanalytical Systems (West Lafayette, IN) LC3D (Petite Ampere) potentiostat was used to apply a potential of + 450 mV relative to a Ag/AgCl reference electrode and a detection current baseline was obtained in approximately 5 min. Then, an aqueous DA solution was injected using a Hamilton (Reno, NV) CR-700-20 constant rate syringe and the resulting current outputs were recorded onto a Tektronix (Beaverton, OR) TDS 1002 digital storage oscilloscope. The initial velocities of DA transport were calculated from the linear slope of the initial apparent zero order portion of a plot of [DA] versus time as described previously (Earles et al., 1998; Volz et al., 2006b).
The resulting vesicular DA transport velocities were fit to either the Michaelis-Menten equation

\[ v = \frac{V_{\text{max}} \cdot [DA]}{K_m + [DA]} \]  

(1)

or the Hill equation

\[ v = \frac{V_{\text{max}} \cdot [DA]^h}{K_{0.5}^h + [DA]^h} \]  

(2)

with non-linear regression using GraphPad Prism V4.0 (San Diego, CA) as previously described (Segel, 1993; Motulsky and Christopoulos, 2003). In these equations, \( v \) is the transport velocity, \( V_{\text{max}} \) is the maximal transport velocity, \( K_m \) is the Michaelis-Menten constant, \([DA]\) is the initial extravesicular concentration of exogenously added DA, \( K_{0.5} \) is formally defined as the substrate concentration at half maximal transport velocity in sigmoidal response curves, and \( h \) is the Hill coefficient. The density of kinetically active VMAT-2, the catalytic rate constant, and the rate constant for DA binding to the VMAT-2 were calculated as previously described (Volz et al., 2006a). Protein concentrations were measured using a BioRad Laboratories (Hercules, CA) Bradford protein assay. Indicators of precision are standard errors of the mean and statistical comparisons of the results were made using a t-test.

The total amount of DA transported via the cytoplasmic and membrane-associated vesicles was determined by using RDE voltammetry to measure the amount of DA transported in the cytoplasmic and membrane-associated vesicle fractions per unit time.
(i.e., pmol DA/s). These values were then normalized to the original wet weight of the tissues used to prepare the vesicle fractions (i.e., pmol DA/(s x g wet weight)). The cytoplasmic and membrane-associated vesicle fractions contained all of the tissue recoverable from two rat striata and thus the amounts of DA that are ultimately partitioned into each vesicle fraction were readily compared despite large differences in the amount of protein in each fraction. Indicators of precision are standard errors of the mean.

Immunoreactivity

After RDE measurement of DA transport velocities, SDS-polyacrylamide gel electrophoresis and western blot analysis was performed on the synaptosome, membrane-associated vesicle, and cytoplasmic vesicle fractions as previously described (Riddle et al., 2002; Sandoval et al., 2002). To compare DAT and VMAT-2 levels in the synaptosome, membrane-associated vesicle, and cytoplasmic vesicle fractions, the pellets were resuspended at 50 mg original striatal wet weight /ml and 80 µl was loaded per well. For analysis of actin and VMAT-2 trafficking in the vesicle fractions, 40 µg of protein from each membrane-associated vesicle sample and 5 µg of protein from each cytoplasmic vesicle sample were used. For analysis of Na⁺/K⁺-ATPase and piccolo in the vesicle fractions, 32 µg of protein from each membrane-associated vesicle sample and 7 µg of protein from each cytoplasmic vesicle sample were utilized. Bound antibody was visualized with horseradish peroxidase-conjugated secondary antibody (rabbit secondary from Biosource (Camarillo, CA) or mouse secondary from Chemicon (Temecula, CA)) and bands on blots were quantified by densitometry using a FluorChem SP Imaging.
System from Alpha Inotech Corp. (San Leandro, CA). Indicators of precision are standard errors of the mean and statistical comparisons of the results were made using a t-test.

Vesicular DA Content

Vesicular DA content was measured by high performance liquid chromatography as described previously (Sandoval et al., 2003; Truong et al., 2005). The P3 (membrane-associated vesicles) and P4 (cytoplasmic vesicles) pellets were prepared as described above and resuspended in ice-cold tissue buffer at 50 and 100 mg original striatal wet weight/ml of tissue buffer, respectively. The resuspended vesicle preparations were then sonicated for ~10 s and centrifuged (22,000 x g for 15 min at 4 °C). A 100 µl aliquot of the resulting supernatant was injected onto a high performance liquid chromatograph system (4.6 mm x 250 mm Whatman International, Ltd. (Maidstone, England) Partisphere C18 column) that was coupled to an electrochemical detector (+ 730 mV relative to a Ag/AgCl reference electrode). The pH 2.86 mobile phase consisted of 50 mM sodium phosphate, 30 mM citric acid, 0.16 mM EDTA, 1.5 mM sodium octyl sulfate, and 10 % (v/v) methanol (Chapin et al., 1986). Indicators of precision are standard errors of the mean and statistical comparisons of the results were made using one way ANOVA with a Tukey post test.

RDE Measurement of K⁺- stimulated DA Release

RDE voltammetry was used to measure K⁺-stimulated DA release in striatal suspensions (Volz et al., 2004; Volz and Schenk, 2004) prepared from treated rats as
previously described (McElvain and Schenk, 1992a). Each sample consisted of one striata (~28 - 38 mg wet weight) that was placed on an ice-cold watch glass and chopped by hand with an ice-cold razor blade for ~30 s. The chopped striatum was then placed in 500 µl of DAT assay buffer inside the RDE glass chamber and was disrupted by repetitive pipetting for approximately 1 min. The resulting striatal suspension was allowed to stand for 12 min and was then washed by the addition and subsequent removal of 250 µl of fresh DAT assay buffer six times. A detection current baseline was obtained as described above in approximately 18 min and then a small quantity of DAT assay buffer containing an elevated KCl concentration (resulting in 40 mM K⁺ inside the RDE glass chamber) was added to the striatal suspension to stimulate DA release (McElvain and Schenk, 1992a). The initial velocity of K⁺-stimulated DA release (obtained from the first 3 s of release) and the magnitude of K⁺-stimulated DA release (taken as the maximum amount of DA released) were calculated as described previously (McElvain and Schenk, 1992a). Indicators of precision are standard errors of the mean and statistical comparisons of the results were made using a t-test.

Drugs and Chemicals

Solutions were made using university-supplied deionized water that was further purified to 18 MΩ with a Diamond Water Purification System from Barnstead (Dubuque, IA). Tetrabenazine was a generous gift from Drs. Jeffrey Erickson and Helene Varoqui (Louisiana State University Health Sciences Center, New Orleans, LA) and was first dissolved in absolute ethanol before being diluted to final concentration in assay buffer as reported previously (Volz et al., 2006b). The ethanol did not affect DA transport in either
the synaptosomal or membrane-associated vesicle fractions (data not shown). (+)-MPD hydrochloride and (-)-cocaine hydrochloride were supplied by the National Institute on Drug Abuse (Bethesda, MD). MPD doses were calculated as the free base and were dissolved in 0.9 % (w/v) saline before being administered at 1 ml/kg as indicated in the figure legends. The pH 7.4 sucrose buffer contained 320 mM sucrose, 3.8 mM NaH₂PO₄, and 12.7 mM Na₂HPO₄ (Sandoval et al., 2001). The pH 7.5 VMAT-2 assay buffer (Erickson et al., 1990; Teng et al., 1997) consisted of 25 mM HEPES, 100 mM potassium tartrate, 0.05 mM EGTA, 0.1 mM EDTA, and 2 mM ATP-Mg⁺² (with the exception of experiments designed to investigate the ATP-dependence of DA transport where the assay buffer contained no ATP-Mg⁺² (Volz et al., 2006b)). The pH 7.4 DAT assay buffer consisted of 126 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 16 mM sodium phosphate, 1.4 mM MgSO₄, and 11 mM dextrose (Sandoval et al., 2001). The pH 2.5 tissue buffer consisted of 50 mM sodium phosphate, 30 mM citric acid, and 10 % (v/v) methanol (Sandoval et al., 2003; Truong et al., 2005). VMAT-2 antibody (AB1767) was purchased from Chemicon (Temecula, CA), Na⁺/K⁺-ATPase antibody from BD Biosciences (San Jose, CA), piccolo antibody from ABCAM (Cambridge, MA), actin antibody from ICN Biotechnologies (Costa Mesa, CA), and DAT antibody was generously provided by Dr. Roxanne Vaughan (University of North Dakota, Grand Forks, ND).
RESULTS

Results presented in Figure 1A confirmed previous findings that a single injection of MPD (40 mg/kg, s.c.; a dose used previously to investigate MPD-induced vesicular trafficking (Sandoval et al., 2002)) redistributes VMAT-2 within nerve terminals, as assessed 1 h after treatment. MPD decreased VMAT-2 immunoreactivity in the membrane-associated vesicle fraction and increased VMAT-2 immunoreactivity in the cytoplasmic vesicle fraction. Actin immunoreactivity, used to demonstrate equal protein loading onto the gels, was not altered by MPD (Figure 1B).

Western blot analysis was conducted to estimate the relative amounts of DAT and VMAT-2 in both vesicle fractions. Virtually all of the DAT isolated from the synaptosomal fraction was present in the membrane-associated vesicle fraction (Figure 2A) while VMAT-2 isolated from the synaptosome was distributed between the cytoplasmic and membrane-associated vesicle fractions (Figure 2B) with the membrane-associated vesicle fraction containing the majority of the VMAT-2 isolated from the synaptosome. The membrane-associated vesicle fraction also contained the plasmalemmal membrane marker, Na⁺/K⁺-ATPase, and the readily releasable/active zone marker, piccolo (data not shown). Neither of these markers were detected under the assay conditions utilized (see Methods) in the cytoplasmic vesicle fraction.

Before characterizing the kinetics of DA transport in the two vesicle populations, the specificity of transport was established. This had been accomplished previously for cytoplasmic vesicles, where vesicular DA transport was demonstrated to be ATP-dependent and blocked by the VMAT-2 inhibitor tetrabenazine (Volz et al., 2006b). In the present study, 60 nM tetrabenazine, a concentration that was without effect on
synaptosomal (i.e., DAT-mediated) DA transport (Figure 3A), completely blocked DA transport in the membrane-associated vesicles (Figure 3B). The absence of ATP likewise inhibited DA transport in the membrane-associated vesicles (Figure 3C). Additionally, 100 µM cocaine completely blocked DAT-mediated synaptosomal DA transport (Figure 4A) but was without effect of DA transport in the membrane associated vesicles (Figure 4B). These results demonstrate that membrane-associated vesicular DA transport is selectively mediated by the VMAT-2 and that the DAT does not contribute to the measured DA transport in this preparation.

Results presented in Figure 5 compare the kinetics of vesicular DA transport in the cytoplasmic and membrane-associated fractions. Figure 5A demonstrates that the relationship between the initial concentrations of DA and the measured DA transport velocities expressed per µg protein was of the expected rectangular hyperbolic shape in cytoplasmic vesicles from both saline- and MPD-treated animals and that the velocities were modeled by the Michaelis-Menten equation (Eq. 1). MPD increased the $V_{\text{max}}$ (3.71 ± 0.03 saline vs. 5.48 ± 0.07 fmol/(s x µg protein) MPD, $p < 0.05$), without affecting the $K_m$ (318 ± 9 saline vs. 303 ± 12 nM MPD) of cytoplasmic vesicular DA transport. Further kinetic analysis indicated that MPD increased the density of kinetically active VMAT-2 (0.36 ± 0.04 saline vs. 0.55 ± 0.02 fmol/µg protein MPD, $p < 0.05$). However, neither the catalytic rate constant (10 ± 1 saline vs. 10.0 ± 0.4 s⁻¹ MPD) nor the rate constant for DA binding to the VMAT-2 (3.2 ± 0.5 x 10⁷ saline vs. 3.3 ± 0.2 x 10⁷ M⁻¹s⁻¹ MPD) were altered by MPD treatment. The total amount of DA transported in the entire cytoplasmic vesicle fraction (see Materials and Methods for description of calculation) measured at 600 nM DA (i.e., a concentration that presumably resembles intracellular
DA levels; see Discussion below) was $6 \pm 3$ pmol/(s x g wet weight) ($N = 4$). The total amount of DA transported in the entire cytoplasmic vesicle fraction measured at $2 \, \mu M$ DA (i.e., a DA concentration observed at the plateau value shown in Figure 5A) was $9 \pm 2$ pmol/(s x g wet weight) ($N = 4$).

In striking contrast to the rectangular hyperbolic curves obtained with cytoplasmic vesicles (Figure 5A), Figure 5B demonstrates that the relationship between the initial concentrations of DA and the measured DA transport velocities expressed per $\mu g$ protein in membrane-associated vesicles from both saline- and MPD-treated animals was sigmoidal, suggesting cooperativity. The velocities depicted in Figure 5B were modeled by the Hill equation (Eq. 2) and the Hill coefficients were similar in both saline- and MPD-treated animals ($4.5 \pm 0.9$ saline vs. $5.4 \pm 0.6$ MPD). Surprisingly, MPD increased DA transport in the membrane-associated vesicles by increasing the $V_{\text{max}}$ ($0.51 \pm 0.04$ saline vs. $0.83 \pm 0.03$ fmol/(s x $\mu g$ protein) MPD, $p < 0.05$) while decreasing VMAT-2 immunoreactivity (Figure 1A). MPD also caused a small but statistically significant decrease in the concentration of DA needed to attain half maximal velocity (designated $K_{0.5}$ to distinguish it from the $K_m$ of Michaelis-Menten kinetics (Segel, 1993; Motulsky and Christopoulos, 2003); $2.1 \pm 0.1$ saline vs. $1.85 \pm 0.05$ $\mu M$ MPD, $p < 0.05$). Further kinetic analysis of rate constants, as was conducted for the cytoplasmic vesicles, was not possible because the membrane-associated vesicles did not obey Michaelis-Menten kinetics. The total amount of DA transported in the entire membrane-associated vesicle fraction (see Materials and Methods for description of calculation) measured at $600 \, nM$ DA (i.e., a concentration that presumably resembles intracellular DA levels; see Discussion below) was $5 \pm 2$ pmol/(s x g wet weight) ($N = 4$). The total DA transport
values for the membrane-associated vesicles as assessed at 2 µM DA (i.e., a DA concentration observed at the plateau value for cytoplasmic vesicles shown in Figure 5A but below the plateau in Figure 5B) and at 4 µM DA (where transport has reached a plateau value in Figure 5B) were 47 ± 4 and 81 ± 11 pmol/(s x g wet weight) (N=4), respectively.

Results presented in Figure 6 demonstrate that MPD administration increased DA content, as assessed ex vivo, in both the cytoplasmic and membrane-associated vesicle fractions. MPD administration increased both the magnitude and velocity of K+-stimulated DA release in striatal suspensions as well (Figure 7). However, MPD administration did not affect the duration of K+-stimulated DA release (6 ± 1 vs. 7.0 ± 0.2 s for saline vs. MPD, respectively (N =4)).
DISCUSSION

Our laboratory reported (Sandoval et al., 2002) that administration of the widely prescribed psychostimulant, MPD, increases DA transport in cytoplasmic vesicles purified from the striata of MPD-treated rats. This increase occurs concurrent with a redistribution of VMAT-2 protein from the membrane-associated fraction to the cytoplasmic fraction. Both phenomena are DA receptor-mediated and could be manipulated pharmacologically, as in vivo pretreatment with either a D1 or a D2 receptor antagonist attenuates these changes and a D2 receptor agonist mimics the effects of MPD (Sandoval et al., 2002; Truong et al., 2004). The present study expands upon these findings by demonstrating that MPD treatment increases the density of kinetically active VMAT-2 in the cytoplasmic vesicle fraction without affecting either the catalytic rate constant (10 s⁻¹) or the rate constant for DA binding to the VMAT-2 (~3 x 10⁷ M⁻¹s⁻¹). These results suggest that the kinetics of DA binding to the VMAT-2 and translocation across the vesicular membrane are unaltered by MPD treatment and that MPD-induced trafficking of VMAT-2 and associated vesicles is solely responsible for the increase in cytoplasmic vesicular DA transport (for review see Volz et al., 2006b and references therein).

In the course of investigating the impact of MPD on DA transport into cytoplasmic vesicles, an additional unique population of vesicles was identified which exhibited prominently distinct properties as discussed below. DA transport in these membrane-associated vesicles was completely inhibited in the presence of tetrabenazine and in the absence of ATP (Figure 3), indicating that the DA transport is mediated exclusively by the VMAT-2. The tetrabenazine-induced inhibition occurred at a
concentration that was without effect on synaptosomal (i.e., DAT-mediated) DA transport (Figure 3A) demonstrating that the DAT in the membrane-associated vesicle fraction (Figure 2A) does not contribute to the observed DA transport. Further supporting this conclusion, DA transport in the membrane-associated vesicles was unaffected by cocaine at a concentration that completely blocked synaptosomal DA transport (Figure 4). Additionally, DA transport by the DAT is Na\(^+\)- and Cl\(^-\)-dependent (McElvain and Schenk, 1992b) and there was virtually no Na\(^+\) or Cl\(^-\) in the VMAT-2 assay buffer. While the possibility that there may be some small amount of DA binding to the DAT present in the membrane-associated vesicle fraction (Figure 2A) cannot be excluded, this negligible amount is not detectable by RDE voltammetry when VMAT-2-mediated DA transport is inhibited (Figure 3B and C) and therefore does not influence the analysis of VMAT-2 kinetics in the membrane-associated vesicle fraction.

Further study of the membrane-associated vesicles revealed several novel findings. First, and in striking contrast to cytoplasmic vesicular DA transport (Figure 5A), initial velocity vs. DA concentration curves for membrane-associated vesicles from both saline- and MPD-treated animals were of an unexpected sigmoidal shape and were modeled by the Hill equation with a Hill coefficient of approximately 5 (Figure 5B). This is inconsistent with Michaelis-Menten kinetics (Segel, 1993; Fersht, 1998) and suggests that, in contrast to cytoplasmic vesicles, the membrane-associated vesicles display a unique positive cooperativity with respect to DA transport. Substrate cooperativity, especially of this large magnitude (Hill coefficient ~5), is uncommon among membrane transport proteins and this is the first report of cooperativity involving a vesicular neurotransmitter transporter.
One important consequence of the cooperativity exhibited by vesicles in the membrane-associated preparation under study is that at intermediate substrate concentrations, the sigmoidal response would provide a much more sensitive control of substrate transport rates than the rectangular hyperbolic response of cytoplasmic vesicles. Cytoplasmic DA concentrations have been estimated (in pheochromocytoma-12 cells) to be in the range of 0.5-1 µM (Perlman and Sheard, 1982). This is near the middle of the rectangular hyperbolic curve of cytoplasmic vesicles (Figure 5A) and in the lower portion of the sigmoidal curve of membrane-associated vesicles (Figure 5B). At these concentrations, total DA transport (see Materials and Methods for description of calculation) via the cytoplasmic and membrane-associated vesicles are comparable (i.e., 6 ± 3 vs. 5 ± 2 pmol/(s x g wet weight), respectively). If the concentration of intraneuronal DA were to rise (i.e., after drug treatment or as a consequence of a particular microenvironment), then DA transport in the cytoplasmic vesicles would be saturated while DA transport in the membrane-associated vesicles would increase dramatically. This allows for the possibility that at least a subpopulation of the membrane-associated vesicles may function as a reserve sequestration capacity or “DA sink” to prevent cytoplasmic DA from rising to aberrant levels.

A second related novel finding of this study is that membrane-associated vesicles are capable, at high DA concentrations, of sequestering in total far more DA than the cytoplasmic vesicles. The maximal amount of DA transported in the entire cytoplasmic vesicle fraction was 9 ± 2 pmol/(s x g wet weight). In contrast, DA transport values for the membrane-associated vesicles ranged from 47 ± 4 (at a DA concentration observed at the plateau value for cytoplasmic vesicles) to 81 ± 11 pmol/(s x g wet weight) (at a DA
concentration observed at the plateau value for membrane-associated vesicles, see Results). Thus, total DA transport via the cytoplasmic and membrane-associated vesicles was comparable at “typical” cytoplasmic DA concentrations of 0.5-1 µM (see above) but membrane-associated vesicles were capable of sequestering, in total, five- to nine-fold more DA than cytoplasmic vesicles at higher DA concentrations. This large DA transport capacity of membrane-associated vesicles further underscores their potential reserve sequestration capacity as discussed above.

A third novel finding of this study is that while MPD treatment traffics VMAT-2 and associated vesicles away from membranes (Figure 1A), it also kinetically upregulates the decreased number of VMAT-2 remaining in the membrane-associated fraction such that a larger quantity of DA is transported (Figure 5B). Consistent with this finding, DA content in the membrane-associated vesicle fraction is also increased (Figure 6). One functional consequence of these data relates to findings that MPD post-treatment protects against the persistent dopaminergic deficits caused by treatment with the psychostimulant, methamphetamine. In particular, it has been suggested that methamphetamine promotes aberrant cytoplasmic DA accumulation and the subsequent formation of DA-associated reactive oxygen species, thus leading to long-term damage (Cubells et al., 1994; Cadet and Brannock, 1998; Fumagalli et al., 1999; Hanson et al., 2004; Volz et al., 2007a; Volz et al., 2007b). Previous studies indicated that MPD prevented this damage by increasing DA sequestration in cytoplasmic vesicles (Sandoval et al., 2003). The present studies expand on these findings by suggesting that the DA sequestration-promoting capacity of MPD is not limited to cytoplasmic vesicles, and that MPD-affected membrane-associated vesicles may serve as an additional, and perhaps higher capacity, "DA sink" with
enhanced ability to afford neuroprotection. As abnormal DA disposition likely contributes to the development of Parkinson’s disease (Cubells et al., 1994; Jenner, 1998), a MPD-induced kinetic upregulation of DA transport in membrane-associated vesicles may afford protection in this disease state as well.

In addition to increasing DA transport velocities and DA content in the membrane-associated vesicle fraction, MPD increased the magnitude and initial velocity of K+ -stimulated DA release in striatal suspensions (Figure 7). Because both the degree of vesicle loading and the speed of neurotransmitter release can influence receptor activation (Liu, 2003), these findings suggest the functional consequence that in the striatum MPD treatment influences quantal synaptic transmission by increasing the rate at which DA receptors are exposed to DA, and perhaps the duration of this effect. The MPD-induced increase in DA release was not due to an inhibition of the DAT by residual MPD introduced by the original subcutaneous injection since: 1) MPD administration did not change the duration of K+ -stimulated DA release (see Results); and 2) the striatal suspensions were washed six times prior to the measurement of DA release to remove any MPD that was introduced by the original subcutaneous injection. Instead, the increase was likely due to the MPD-induced kinetic upregulation of, and enhanced DA sequestration afforded by, VMAT-2.

The membrane-associated vesicles co-fractionate with synaptosomal membranes after osmotic lysis: this is supported by the observations that plasmalemmal membrane marker, Na+/K+-ATPase, the DAT, and the readily releasable/active zone marker, piccolo, are among the proteins found in this fraction. Taken together with previous findings that K+-stimulated DA release in rat striatal suspensions is both temperature- and Ca++-
dependent (McElvain and Schenk, 1992a), this suggests that the membrane-associated vesicle fraction may contain the readily releasable population of vesicles. Whether this population contains additional pools of vesicle remains to be determined. Future studies will further address this possibility.

In summary, this is the first report of membrane-associated VMAT-2-containing synaptic vesicles that: 1) have large positive substrate cooperativity; 2) are capable of sequestering greater amounts of DA than cytoplasmic vesicles under conditions of elevated concentrations of DA; and 3) are both kinetically and functionally upregulated by MPD. Vesicles of the membrane-associated fraction are likely of importance as regulators of both intraneuronal DA, and of vesicular DA-release capacity. Because these vesicles can be regulated pharmacologically, they may provide an intriguing target for understanding and treating disorders involving abnormal DA transmission including drug abuse, Parkinson’s disease, and attention-deficit hyperactivity disorder.
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LEGENDS FOR FIGURES

Figure 1. MPD redistributes VMAT-2 immunoreactivity. Rats received a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.) and were killed 1 h later. Panel A demonstrates that MPD decreases VMAT-2 immunoreactivity in membrane-associated vesicles and increases VMAT-2 immunoreactivity in cytoplasmic vesicles. Each column represents the average of four independent densitometric determinations and asterisks indicate a statistical difference, \( p < 0.05 \), between immunoreactivity in saline- and MPD-treated animals. Molecular mass, in kDa, is shown to the side of the representative VMAT-2 blots. The representative actin blots in Panel B demonstrate that MPD does not change actin immunoreactivity in either the membrane-associated or cytoplasmic vesicles (densitometric data not shown).

Figure 2. Virtually all of the DAT isolated from the synaptosome is present in the membrane-associated vesicle fraction while VMAT-2 isolated from the synaptosome is distributed between the cytoplasmic and membrane-associated vesicle fractions. Panels A and B depict DAT and VMAT-2 immunoreactivity, respectively, in the synaptosomal (synap), membrane-associated vesicle (memb), and cytoplasmic vesicle (cyto) subcellular fractions. Each column represents the average of four independent densitometric determinations and molecular mass, in kDa, is shown to the side of the representative blots. ND signifies DAT levels that were not detectable.

Figure 3. DA transport in the membrane-associated vesicles is ATP-dependent and is blocked by tetrabenazine. Each panel depicts representative DA concentration vs. time...
clearance profiles of exogenously applied DA. DA transport in the clearance profiles is indicated by a downward sloping line as DA is transported into synaptosomes or vesicles where the RDE can not detect it. Panel A demonstrates that 60 nM tetrabenazine does not block DAT-mediated DA transport in striatal synaptosomes when added 30 s before DA. Panel B demonstrates that 60 nM tetrabenazine completely blocks DA transport in membrane-associated vesicles when added 30 s before DA. Panel C demonstrates that DA transport in membrane-associated vesicles is ATP-dependent.

Figure 4. DA transport in the membrane-associated vesicles is not blocked by cocaine. As in Figure 3, each panel depicts representative DA concentration vs. time clearance profiles of exogenously applied DA. Panel A demonstrates that 100 µM cocaine completely blocks DAT-mediated DA transport in striatal synaptosomes when added 30 s before DA. Panel B demonstrates that 100 µM cocaine does not block DA transport in membrane-associated vesicles when added 30 s before DA.

Figure 5. DA transport displays Michaelis-Menten kinetics in cytoplasmic vesicles and non-Michaelis-Menten cooperativity in membrane-associated vesicles. Rats received a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.) and were killed 1 h later. Each datum point represents the average of four independent determinations and asterisks indicate a statistical difference, $p < 0.05$, between DA transport velocities in saline- and MPD-treated animals. Panel A demonstrates that MPD increases DA transport velocities in cytoplasmic vesicles. The solid lines represent the best fits of the Michaelis-Menten equation (Eq. 1) to the observed data with $r^2$ values of
0.9985 (saline) and 0.9971 (MPD). Panel B demonstrates that MPD also increases DA transport velocities in membrane-associated vesicles. The solid lines represent the best fits of the Hill equation (Eq. 2) to the observed data with $r^2$ values of 0.9736 (saline) and 0.9876 (MPD). DA transport velocities at the lowest concentrations of DA are magnified in the insert.

Figure 6. MPD increases DA content in both the cytoplasmic and membrane-associated vesicle fractions. Rats received a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.) and were killed 1 h later. Each column represents the average of six independent determinations and asterisks indicate a statistical difference, $p < 0.05$, between DA content in saline- and MPD-treated animals.

Figure 7. MPD increases both the magnitude and velocity of K\(^+\)-stimulated DA release. Rats received a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.) and were killed 1 h later. Each column represents the average of three independent determinations and asterisks indicate a statistical difference, $p < 0.05$, between the magnitude and velocity of K\(^+\)-stimulated DA release in saline- and MPD-treated animals.
A

VMAT-2

membrane-associated vesicles cytoplasmic vesicles

\[
\begin{align*}
\text{band density (arbitrary units)} & \\
0 & \quad 50 \quad 100 \quad 150 \quad 200 \quad 250 \quad 300 \quad 350
\end{align*}
\]

- saline
- MPD

* indicates significant difference

B

actin

membrane-associated vesicles cytoplasmic vesicles

\[
\begin{align*}
\text{size (kDa)} & \\
36 & \quad 50
\end{align*}
\]

- saline
- MPD

FIGURE 1
FIGURE 3

A. Control vs. 60 nM tetrabenazine

1 μM DA

60 nM tetrabenazine

45 s

200 nM DA

B. Control vs. 60 nM tetrabenazine

C. 1.8 mM ATP vs. no ATP

200 nM DA

1.8 mM ATP

no ATP

45 s
FIGURE 4

Panel A: 1 μM DA
- Control
- 100 μM cocaine
- 45 s

Panel B: 200 nM DA
- Control
- 100 μM cocaine
- 45 s
FIGURE 6

Comparison of DA content in membrane-associated vesicles and cytoplasmic vesicles between saline and MPD conditions.