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TITLE PAGE

METHYLPHENIDATE-INDUCED INCREASES IN VESICULAR DOPAMINE

SEQUESTRATION AND DOPAMINE RELEASE IN THE STRIATUM: THE ROLE OF

MUSCARINIC AND DOPAMINE D2 RECEPTORS

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methylphenidate; NMDA, N-methyl-D-aspartate; OXO, oxotremorine; QUIN, quinpirole; SCOP,

scopolamine; RDE, rotating disk electrode; VMAT-2, vesicular monoamine transporter-2

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ABSTRACT

Methylphenidate (MPD) administration alters the subcellular distribution of vesicular monoamine transporter-2 (VMAT-2)-containing vesicles in rat striatum. This report reveals previously undescribed pharmacological features of MPD by elucidating its receptor-mediated effects on VMAT-2-containing vesicles that co-fractionate with synaptosomal membranes after osmotic lysis (referred to herein as membrane-associated vesicles) and on striatal dopamine (DA) release. MPD administration increased DA transport into, and decreased the VMAT-2 immunoreactivity of, the membrane-associated vesicle subcellular fraction. These effects were mimicked by the D2 receptor agonist, quinpirole, and blocked by the D2 receptor antagonist, eticlopride. Both MPD and quinpirole increased vesicular DA content. However, MPD increased, whereas quinpirole decreased, K^+-stimulated DA release from striatal suspensions. Like MPD, the muscarinic receptor agonist, oxotremorine, increased K^+-stimulated DA release. Both eticlopride and the muscarinic receptor antagonist, scopolamine, blocked MPD-induced increases in K^+-stimulated DA release while the N-methyl-D-aspartate receptor antagonist, MK-801, was without effect. This suggests that D2 receptors mediate both the MPD-induced redistribution of vesicles away from synaptosomal membranes and the MPD-induced upregulation of vesicles remaining at the membrane. This results in a redistribution of DA within the striatum from the cytoplasm into vesicles, leading to increased DA release. However, D2 receptor activation alone is not sufficient to mediate the MPD-induced increases in striatal DA release as muscarinic receptor activation is also required. These novel findings provide insight into the mechanism of action of MPD, regulation of DA sequestration/release, and treatment of disorders affecting DA disposition including attention-deficit hyperactivity disorder, substance abuse, and Parkinson's disease.

INTRODUCTION

The ritalinic acid psychostimulant, methylphenidate (MPD), is frequently used to treat attention-deficit hyperactivity disorder. It is well established that MPD prevents the clearance of dopamine (DA) from the synaptic cleft by binding to the neuronal DA transporter (DAT) (Wayment et al., 1999; Volz et al., 2005; Volz and Schenk, 2005). In addition, MPD also indirectly affects the vesicular monoamine transporter-2 (VMAT-2); a protein that sequesters cytoplasmic DA inside the synaptic vesicles of nerve terminals. A single MPD treatment increases DA transport, VMAT-2 immunoreactivity, and binding of the VMAT-2 ligand, [^{3}H]dihydrotetrabenazine, in cytoplasmic vesicles purified from osmotic lysates of rat striatal synaptosomes (Sandoval et al., 2002; Sandoval et al., 2003; Volz et al., 2007a). These effects on cytoplasmic vesicles are D2 receptor-mediated, as the D2 receptor antagonist, eticlopride (ETIC), attenuates or blocks these effects and the D2 receptor agonist, quinpirole (QUIN), mimics the effects of MPD (Sandoval et al., 2002; Truong et al., 2004).

In contrast to studies described above involving cytoplasmic vesicles, a recent study (Volz et al., 2007a) characterized the largely undescribed population of membrane-associated VMAT-2-containing vesicles that co-fractionate with striatal synaptosomal membranes after osmotic lysis. Strikingly and in contrast to cytoplasmic vesicles, DA transport into these membrane-associated vesicles is cooperative with a sigmoidal response curve and has a large DA sequestration capacity at elevated concentrations of DA (Volz et al., 2007a). As predicted from studies involving cytoplasmic vesicles, MPD administration decreases VMAT-2 immunoreactivity in this membrane-associated vesicle fraction. Unexpectedly, MPD also kinetically upregulates DA transport in vesicles remaining in the membrane-associated fraction after MPD-induced trafficking such that these vesicles sequester a larger quantity of DA due to

an increase in the rate at which the VMAT-2 transports DA (Volz et al., 2007a). As a result, MPD also increases exocytotic DA release (Volz et al., 2007a).

While the impact of D2 receptors on cytoplasmic vesicles has been reported (Sandoval et al., 2002; Truong et al., 2004), the receptor-mediated mechanisms underlying the effects of MPD on membrane-associated vesicle function have not been described. Accordingly, this report presents new information elucidating the receptor-mediated mechanisms responsible for the effects of MPD on this important membrane-associated vesicle population using ETIC and QUIN and also describes the functional consequences of these effects. Owing to the affinity of MPD for muscarinic receptors (Markowitz et al., 2006), the effects of the muscarinic receptor antagonist, scopolamine (SCOP), and the muscarinic receptor agonist, oxotremorine (OXO), on DA release were also examined. In addition, because the anatomical localization of muscarinic receptors suggests the possibility that muscarinic receptors may influence glutamatergic function (Hersch et al., 1994) and high concentrations of glutamate can increase striatal DA release (Moghaddam et al., 1990), the effects of the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 ((5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), were also studied. The results demonstrate the new findings that D2 receptor activation mediates the MPD-induced upregulation of DA transport in vesicles remaining at the membrane and a MPD-induced increase in striatal DA release. Muscarinic, but not NMDA, receptor activation is also required for MPD to affect the actual DA release process. These findings provide novel insights into the receptor-mediated mechanism of action of MPD in the striatum as well as the physiological regulation of vesicular DA sequestration and synaptic transmission. Accordingly, these data may advance the treatment of disorders involving abnormal DA disposition including Parkinson's disease, attention-deficit hyperactivity disorder, and substance abuse.

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METHODS

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). VMAT-2

antibody was purchased from Chemicon (Temecula, CA). (±)-MPD hydrochloride was supplied

by the Research Triangle Institute (Research Triangle Park, NC). ETIC hydrochloride, MK-801

maleate, and OXO sesquifumarate salts were purchased from Sigma (St. Louis, MO). QUIN

hydrochloride and SCOP hydrobromide were purchased from Tocris (Ellisville, MO). All drugs

were administered at doses previously used to investigate receptor-mediated effects in rat brain

(Hernandez-Lopez et al., 1992; Keefe and Adams, 1998; Casas et al., 1999; Forster and Blaha,

2000; Ichikawa et al., 2002; Sandoval et al., 2002; Kilbourn et al., 2004; Truong et al., 2004;

Riddle et al., 2007; Volz et al., 2007a). Drug 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 NaH_2PO_4, and 12.7 mM

Na_2HPO_4. The pH 7.5 VMAT-2 assay buffer consisted of 25 mM HEPES, 100 mM potassium

tartrate, 0.05 mM EGTA, 0.1 mM EDTA, and 2 mM ATP-Mg^{+2}. The pH 7.4 DAT assay buffer

consisted of 126 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 16 mM sodium phosphate, 1.4 mM

$MgSO_4$, and 11 mM dextrose. The pH 2.5 tissue buffer consisted of 50 mM sodium phosphate,

30 mM citric acid, and 10 % (v/v) methanol.

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 approved by the University of Utah Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Subcellular Fractionation and Measurement of DA Transport Velocities

Rotating Disk Electrode (RDE) voltammetry (Schenk et al., 2005; Volz et al., 2006) was used to measure the initial velocities of inwardly directed vesicular DA transport into membrane-associated vesicles purified from rat striata as described previously (Volz et al., 2007a). 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 ^{o}C) to remove nuclear debris. The resulting supernatant (S1) was centrifuged (22,000 x g for 15 min at 4 ^{o}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 ^{o}C) to form a pellet (P3), which contained the membrane-associated vesicles, and a supernatant (S3). To isolate cytoplasmic vesicles, 1 mM ice-cold (pH 7.5) $MgSO_4$ was added to the S3 supernatant and the resulting mixture was centrifuged (100,000 x g for 45 min at 4 ^{o}C) to obtain the cytoplasmic vesicle pellet (P4).

To measure DA transport velocities, the P3 membrane-associated vesicle pellet was resuspended in 500 μl of VMAT-2 assay buffer and 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. 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, 10.2 μl of an aqueous DA solution was injected using a Hamilton (Reno, NV) CR-700-20 constant rate syringe which resulted in 1.5 μM DA inside chamber. The current outputs were recorded onto a Tektronix (Beaverton, OR) TDS 1002 digital storage oscilloscope and 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 (Volz et al., 2006). Protein concentrations were measured using a BioRad Laboratories (Hercules, CA) Bradford protein assay.

VMAT-2 Immunoreactivity

After RDE measurement of DA transport velocities, SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed on the membrane-associated vesicle samples as previously described (Riddle et al., 2002; Volz et al., 2007a). Equal amounts of protein (40 μg of protein as determined by a BioRad Laboratories (Hercules, CA) Bradford protein assay) from each membrane-associated vesicle sample were loaded onto the gel. Bound

VMAT-2 antibody was visualized with horseradish peroxidase-conjugated secondary antibody (rabbit secondary from Biosource (Camarillo, CA)) and bands on blots were quantified by densitometry using a FluorChem SP Imaging System from Alpha Inotech Corp. (San Leandro, CA).

Vesicular DA Content

Vesicular DA content was measured by high performance liquid chromatography as described previously (Volz et al., 2007a). 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 ^{o}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).

Measurement of K^+- stimulated DA Release

RDE voltammetry (Schenk et al., 2005; Volz et al., 2006) was used to measure K^+-stimulated DA release in striatal suspensions prepared from treated rats as previously described (McElvain and Schenk, 1992; Volz et al., 2007a). Each sample consisted of one striatum (~ 28-38 mg wet weight) that was placed on an ice-cold watch glass and chopped by hand with an ice-

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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 (done without disturbing the settled striatal tissue) 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. The initial velocity of K^+-stimulated DA release

(obtained from the first 3 s of release), the magnitude of K^+-stimulated DA release (taken as the

maximum amount of DA released), and the duration of K^+-stimulated DA release (the time

required for the maximum amount of DA to be released) were calculated and normalized to

striatal wet weight as described previously (McElvain and Schenk, 1992; Volz et al., 2007a).

RESULTS

Experiments were initially conducted to investigate the D2 receptor-mediated effects of MPD on membrane-associated vesicles. In these studies, it is important to note that the plasmalemmal DAT does not contribute to measurable DA binding or transport in the membrane-associated vesicle subcellular fraction (Volz et al., 2007a). The results presented in Fig. 1 confirmed earlier findings (Volz et al., 2007a) that a single administration of MPD (40 mg/kg, s.c.; a dose used previously to characterize the impact of MPD on cytoplasmic vesicles (Sandoval et al., 2002; Volz et al., 2007a)) increases DA transport velocities (Fig. 1A). MPD (40 mg/kg, s.c.) also concurrently decreases VMAT-2 immunoreactivity (Fig. 1B) without altering actin immunoreactivity (Volz et al., 2007a) in the membrane-associated vesicle fraction 1 h after treatment. Both the ability to increase DA transport velocities and to decrease VMAT-2 immunoreactivity were completely blocked by pretreatment with the D2 receptor antagonist, ETIC (0.5 mg/kg, i.p.). The D2 receptor agonist, QUIN (1 mg/kg, i.p.), mimicked the effects of MPD (40 mg/kg, s.c.) by increasing DA transport velocities (Fig. 2A) and decreasing VMAT-2 immunoreactivity (Fig. 2B) in the membrane-associated vesicle fraction. These effects of QUIN (1 mg/kg, i.p.) were blocked by pretreatment with ETIC (0.5 mg/kg, i.p.).

The role of D2 receptors in mediating the effects of MPD on vesicular DA content and K^+-stimulated DA release was then examined. As shown in Fig. 3, MPD administration (40 mg/kg, s.c.) increased DA content in both the cytoplasmic and membrane-associated vesicle fractions. QUIN (1 mg/kg, i.p.) mimicked the effects of MPD (40 mg/kg, s.c.) by also increasing DA content in both the cytoplasmic and membrane-associated vesicle fractions (Fig. 4). MPD administration (40 mg/kg, s.c.) increased both the velocity (Fig. 5A) and magnitude (Fig. 5B) of K^+-stimulated DA release from striatal suspensions. Both effects of 40 mg/kg MPD were

completely blocked by pretreatment with ETIC (0.5 mg/kg, i.p.). Neither MPD (40 mg/kg, s.c.) nor ETIC (0.5 mg/kg, i.p.) affected the duration of K^+-stimulated DA release (8 ± 1 s for saline/saline, 8.2 ± 0.7 s for saline/MPD, 8.7 ± 0.4 s for ETIC/saline, and 8.9 ± 0.4 s for ETIC/MPD; N = 4).

In striking contrast to the D2 receptor-mediated ability of MPD to *increase* DA release, QUIN administration (1 mg/kg, i.p.) *decreased* both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions (Fig. 6). QUIN (1 mg/kg, i.p.) did not affect the duration of K^+-stimulated DA release (5 ± 1 s for saline and 5.4 ± 0.2 s for QUIN; N = 4). Given this apparent disparity between these D2 receptor-mediated effects on DA release and numerous reports demonstrating that D2 receptor activation decreases K^+-stimulated striatal DA release (Starke et al., 1989; Pothos et al., 1998), additional experiments were conducted to determine the mechanism underlying the effects of MPD on DA release. MPD has affinity for muscarinic receptors (Markowitz et al., 2006) with a reported K_i for rat central nervous system muscarinic receptors (non-subtype-selective) of 3.2 μM (data obtained from the NIDA Addiction Treatment Discovery Program through contract # N01DA-8-8089 with NovaScreen Biosciences Corporation (Hanover, MD)). Thus, the role of muscarinic receptors in mediating the effects of MPD on DA release was investigated.

Consistent with a role for muscarinic receptors in mediating MPD-induced DA release, administration of the muscarinic receptor agonist, OXO (1.5 mg/kg, s.c.), mimicked the effects of MPD by increasing both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions (Fig. 7). OXO (1.5 mg/kg, s.c.) did not affect the duration of K^+-stimulated DA release (6.1 ± 0.2 s for saline and 6.4 ± 0.2 s for OXO; N = 4). A single administration (5 mg/kg, i.p.) of the muscarinic receptor antagonist, SCOP, did not alter the ability of a high dose of MPD

(40 mg/kg, s.c.) to increase K^+-stimulated DA release (data not shown). However, SCOP (5 mg/kg, i.p.) completely blocked the ability of a lower dose of MPD (2 mg/kg, s.c.; a dose also demonstrated to cause vesicle trafficking (Riddle et al., 2007)) to increase the velocity (Fig. 8A) and magnitude (Fig. 8B) of K^+-stimulated DA release. Neither 2 mg/kg MPD nor 5 mg/kg SCOP affected the duration of K^+-stimulated DA release (6.5 ± 0.6 s for saline/saline, 7.2 ± 0.4 s for saline/MPD, 7.2 ± 0.4 s for SCOP/saline, and 7.0 ± 0.8 s for SCOP/MPD; N = 4).

Finally, because the anatomical localization of muscarinic receptors suggests the possibility that muscarinic receptors may influence glutamatergic function (Hersch et al., 1994) and high concentrations of glutamate can increase striatal DA release (Moghaddam et al., 1990), the effects of the NMDA receptor antagonist, MK-801, were also studied. A single administration (1 mg/kg, i.p.) of MK-801 did not alter the ability of the low dose of MPD (2 mg/kg, s.c.) to increase K^+-stimulated release velocity (Fig. 9A) and magnitude (Fig. 9B). Neither 1 mg/kg (i.p.) MK-801 nor 2 mg/kg MPD (s.c.) affected the duration of K^+-stimulated DA release (7.2 ± 0.5 s for saline/saline, 7.2 ± 0.4 s for saline/MPD, 7.5 ± 0.4 s for MK-801/saline, and 8.0 ± 0.5 s for MK-801/MPD; N = 4).

DISCUSSION

The present study confirms and extends previous findings involving cytoplasmic vesicles (Sandoval et al., 2002; Truong et al., 2004) that D2 receptors mediate the ability of MPD to traffic synaptosomal membrane-associated synaptic vesicles from membranes into the cytoplasm as ETIC blocks the MPD-induced decreases in membrane-associated VMAT-2 immunoreactivity (Fig. 1). ETIC also prevents the MPD-induced increase in vesicular DA transport. Like MPD, QUIN increases DA transport and decreases VMAT-2 immunoreactivity in the membrane-associated vesicle fraction, and these effects are blocked by ETIC (Fig. 2). Taken together, these data suggest that both the MPD-induced trafficking of vesicles out of the membrane-associated fraction and the MPD-induced kinetic upregulation of VMAT-2 in the remaining membrane-associated vesicles are D2 receptor-mediated. However, it should also be noted that QUIN also has some affinity for D3 receptors (Moreland et al., 2004). Because MPD has no significant binding affinity for DA receptors (Markowitz et al., 2006), these effects are likely due to MPD-induced increases in extracellular DA concentrations caused by DAT blockade rather than to a direct interaction between MPD and D2 receptors.

The MPD-induced increases in vesicular DA transport (due to vesicle trafficking in cytoplasmic vesicles and kinetic upregulation of VMAT-2 in membrane-associated vesicles (Volz et al., 2007a)) result in an increase in the DA content of the cytoplasmic and membrane-associated vesicle fractions (Fig. 3). Because MPD administration does not change whole striatal tissue DA content (Sandoval et al., 2003), this likely represents a redistribution of DA within the striatum from the cytoplasm and into the vesicles. Consistent with the above finding that D2 receptors mediate both trafficking and kinetic upregulation, QUIN also increases cytoplasmic and membrane-associated vesicular DA content (Fig. 4).

One functional consequence of MPD-induced increases in vesicular DA sequestration involves the ability of MPD to protect against the persistent dopaminergic deficits caused by methamphetamine (Sandoval et al., 2003). The MPD-induced redistribution of DA within the striatum from the cytoplasm into the vesicles may attenuate methamphetamine-induced aberrant cytoplasmic DA accumulation and the subsequent formation of DA-associated reactive oxygen species (Volz et al., 2007b; Volz et al., 2007c) which cause persistent dopaminergic deficits (Cubells et al., 1994; Cadet and Brannock, 1998; Fumagalli et al., 1999). Because abnormal cytoplasmic DA accumulation may also contribute to the development of Parkinson's disease (Jenner, 1998), the potential of MPD-induced increases in vesicular DA sequestration to attenuate the disease's progression merits further investigation; as suggested by findings that MPD treatment improves motor function in Parkinson's patients (Devos et al., 2007).

Another predictable functional consequence of a MPD-induced increase in the DA content of cytoplasmic and membrane-associated vesicles is an increase in both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions (Volz et al., 2007a). This is of particular interest, as both the speed of neurotransmitter release and the amount of vesicular DA content can influence receptor activation (Liu, 2003; Edwards, 2007), and thus MPD treatment may influence quantal synaptic transmission in the striatum by increasing the rate at which DA receptors are exposed to DA, and perhaps the magnitude and/or duration of this effect. D2 receptor activation mediates these increases, as ETIC blocks both the MPD-induced increases in velocity (Fig. 5A) and magnitude (Fig. 5B) of K^+-stimulated DA release (presumably by blocking the D2 receptor-mediated increases in DA transport and resulting increases in vesicular DA content caused by MPD).

A role for D2 receptor activation in mediating the MPD-induced *increase* in K^+-stimulated DA release is seemingly inconsistent with literature reports indicating that D2 receptor activation *decreases* K^+-stimulated DA release (Starke et al., 1989; Pothos et al., 1998), as well as the present findings that QUIN decreases both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions (Fig. 6). These results suggest that while D2 receptors mediate the effects of MPD on vesicle trafficking, vesicular DA transport, and vesicular DA content, D2 receptors do not wholly and completely mediate the effects of MPD on K^+-stimulated DA release. Thus, investigations were conducted to determine if additional mechanisms contribute to the MPD-induced increases in K^+-stimulated DA release.

Muscarinic receptors were selected for study because MPD has significant affinity for these receptors (see Results). A potential role for muscarinic receptors in mediating the effects of MPD on DA release was suggested by findings that, like MPD, the muscarinic receptor agonist, OXO, increases K^+-stimulated DA release in rat striatum (Raiteri et al., 1984). This potential was confirmed by findings that OXO mimicked the effects of MPD by increasing both the velocity (Fig. 7A) and magnitude (Fig. 7B) of K^+-stimulated DA release from striatal suspensions. Accordingly, experiments were conducted to determine whether antagonism of muscarinic receptors could block the effects of MPD on DA release in the striatum.

SCOP (administered at a commonly used dose of 5 mg/kg (Hernandez-Lopez et al., 1992; Casas et al., 1999; Forster and Blaha, 2000; Kilbourn et al., 2004)) did not alter MPD-induced increases in K^+-stimulated DA release following administration of a high dose (40 mg/kg) of MPD (i.e., the dose selected for study previously when characterizing mechanisms affecting cytoplasmic vesicles (Sandoval et al., 2002; Volz et al., 2007a)). However, SCOP completely blocked the effects of a lower dose (2 mg/kg) of MPD on K^+-stimulated DA release velocity (Fig.

8A) and DA release magnitude (Fig. 8B). It is noteworthy that the lower MPD dose traffics synaptic vesicles (Riddle et al., 2007) and increases the velocity (Fig. 8A) and magnitude (Fig. 8B) of DA release as does the 40 mg/kg dose of MPD , and thus it is likely that both doses elicit the same phenomenon. The inability of SCOP to attenuate the effects of the higher MPD dose may be due to an inability to adequately compete for muscarinic receptor binding sites. Alternatively, non-muscarinic mechanisms may be initiated by the higher dose of MPD that, like muscarinic receptor activation, increase DA release. This latter possibility remains to be tested.

The muscarinic effect on DA release may be indirect and one possible mechanism involves muscarinic receptors controlling glutamate release. The anatomical localization of muscarinic receptors suggests the possibility that muscarinic receptors may influence glutamatergic function (Hersch et al., 1994). Further high concentrations of glutamate can increase striatal DA release (Moghaddam et al., 1990). However, MK-801 pretreatment did not alter the effects of MPD on K^+-stimulated DA release nor did it have any effect on DA release on its own (Fig. 9). This suggests that NMDA receptors do not mediate the increase in DA release seen after MPD which would indicate that changes in NMDA receptor-mediated glutamatergic signaling are not the intermediate step between muscarinic receptor activation and increases in DA release.

In summary, the present results elucidate a heretofore-unreported mechanism whereby MPD alters striatal DA transmission. D2 receptors mediate the ability of MPD to: 1) traffic vesicles away from synaptosomal membranes and into the cytoplasm which increases DA transport into cytoplasmic vesicles; and 2) kinetically upregulate VMAT-2 in vesicles that remain associated with synaptosomal membranes such that DA transport into the membrane-associated vesicles is increased as well. This results in a redistribution of DA within the striatum

from the cytoplasm and into vesicles and a consequent increase in DA release. The present studies also demonstrate that muscarinic receptor, but not NMDA receptor, activation is required for MPD to affect the actual DA release process. This is among the first demonstrations of a role for muscarinic receptors in mediating the effects of MPD. Of interest are recent findings that decreased striatal extracellular DA concentrations may contribute to inattention in adult patients with attention-deficit hyperactivity disorder (Volkow et al., 2007). Thus, in addition to therapeutic potential in treatment of Parkinson's disease and substance abuse, the muscarinic and D2 receptor-mediated effects of MPD on DA disposition may have therapeutic relevance to the treatment of attention-deficit hyperactivity disorder.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. The D2 receptor antagonist, ETIC, blocks MPD-induced increases in DA transport velocity (Panel A) and decreases in VMAT-2 immunoreactivity (Panel B) in the membrane-associated vesicle fraction. Rats received a single administration of ETIC (0.5 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) 15 min before a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.). All animals were killed 1 h after the last injection. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a one-way ANOVA with a Tukey post-test, from other-treated groups. Molecular mass, in kilodaltons, is shown to the side of the representative blot.

Fig. 2. The D2 receptor antagonist, ETIC, blocks the increases in DA transport velocity (Panel A) and decreases in VMAT-2 immunoreactivity (Panel B) in the membrane-associated vesicle fraction caused by the D2 receptor agonist, QUIN. Rats received a single administration of ETIC (0.5 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) 15 min before a single administration of QUIN (1 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.). All animals were killed 1 h after the last injection. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a one-way ANOVA with a Tukey post-test, from other-treated groups. Molecular mass, in kilodaltons, is shown to the side of the representative blot.

Fig. 3. 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 mean $\pm$ SEM of six

independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a two-tailed t test, between DA content in saline- and MPD-treated animals.

Fig. 4. The D2 agonist, QUIN, increases DA content in both the cytoplasmic and membrane-associated vesicle fractions. Rats received a single administration of QUIN (1 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) and were killed 1 h later. Each column represents the mean $\pm$ SEM of six independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a two-tailed t test, between DA content in saline- and QUIN-treated animals.

Fig. 5. The D2 receptor antagonist, ETIC, blocks MPD-induced increases in both the velocity (Panel A) and magnitude (Panel B) of K^+-stimulated DA release from striatal suspensions. Rats received a single administration of ETIC (0.5 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) 15 min before a single administration of MPD (40 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.). All animals were killed 1 h after the last injection. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a one-way ANOVA with a Tukey post-test, from other-treated groups.

Fig. 6. The D2 receptor agonist, QUIN, decreases both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions. Rats received a single administration of QUIN (1 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) and were killed 1 h later. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a two-tailed t test, between DA release in saline- and QUIN-treated animals.

Fig. 7. The muscarinic receptor agonist, OXO, increases both the velocity and magnitude of K^+-stimulated DA release from striatal suspensions. Rats received a single administration of OXO (1.5 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.) and were killed 1 h later. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a two-tailed t test, between DA release in saline- and OXO-treated animals.

Fig. 8. The muscarinic receptor antagonist, SCOP, blocks MPD-induced increases in both the velocity (Panel A) and magnitude (Panel B) of K^+-stimulated DA release from striatal suspensions. Rats received a single administration of SCOP (5 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) 15 min before a single administration of MPD (2 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.). All animals were killed 1 h after the last injection. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference, $p \leq 0.05$ via a one-way ANOVA with a Tukey post-test, from other-treated groups.

Fig. 9. The NMDA receptor antagonist, MK-801, does not block MPD-induced increases in both the velocity (Panel A) and magnitude (Panel B) of K^+-stimulated DA release from striatal suspensions. Rats received a single administration of MK-801 (1 mg/kg, i.p.) or saline vehicle (1 ml/kg, i.p.) 30 min before a single administration of MPD (2 mg/kg, s.c.) or saline vehicle (1 ml/kg, s.c.). All animals were killed 1 h after the last injection. Each column represents the mean $\pm$ SEM of four independent determinations and an asterisk indicates a statistical difference,

$p \leq 0.05$ via a one-way ANOVA with a Tukey post-test, between MPD-treated and saline-treated

groups.

Figure 1

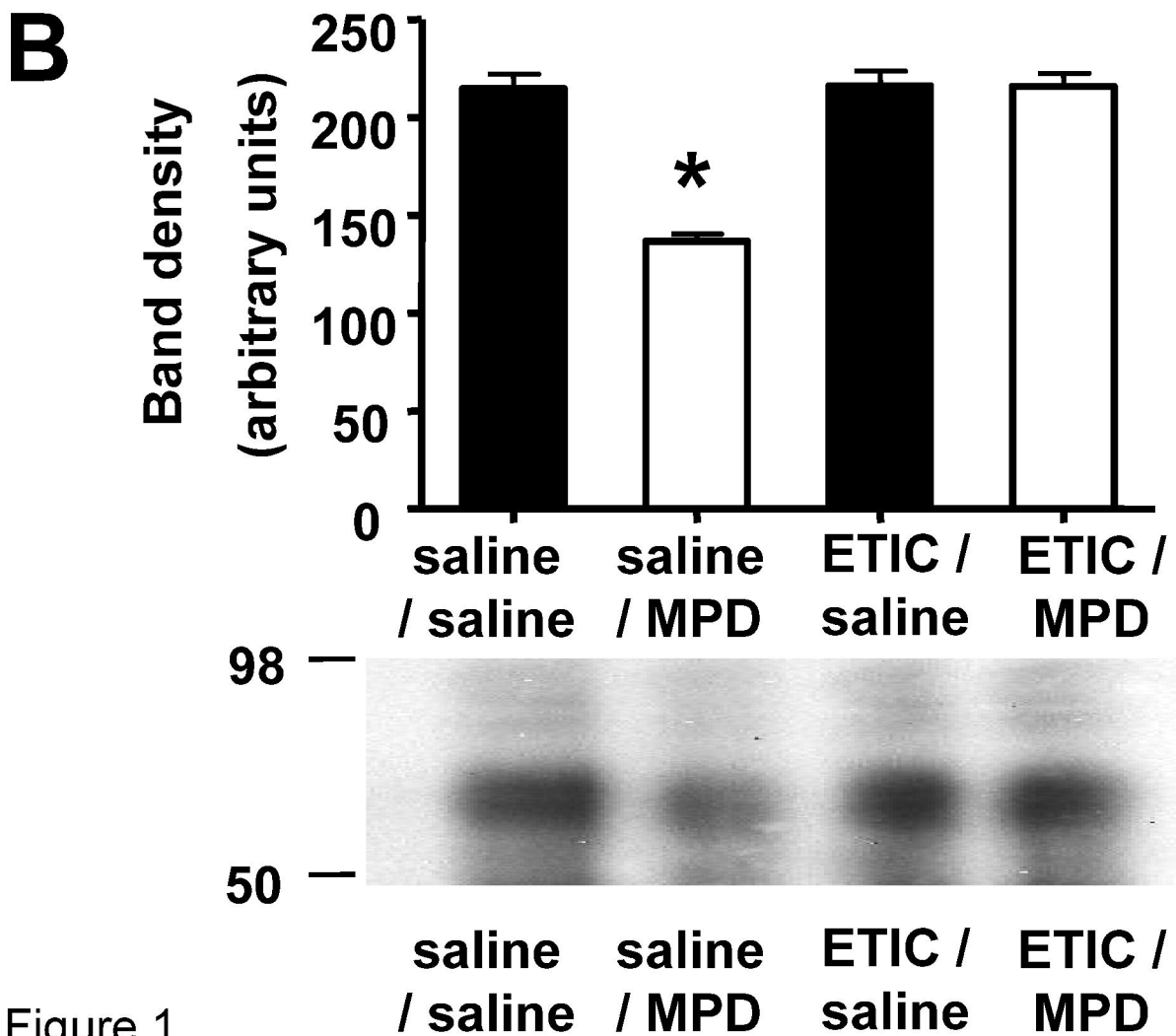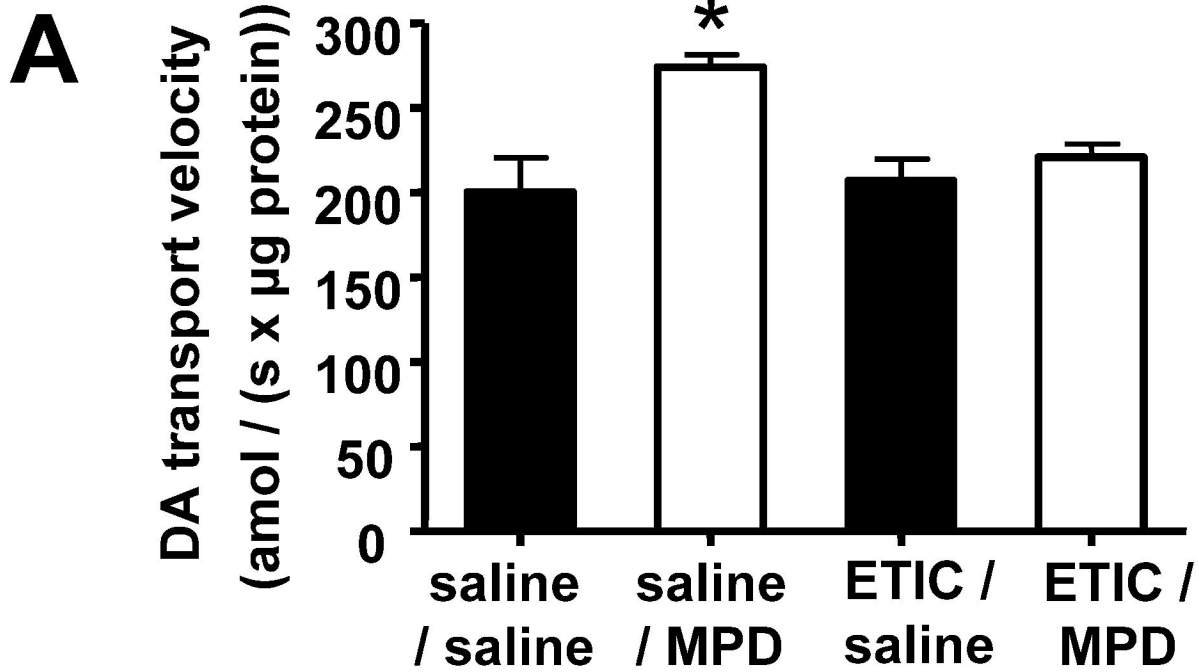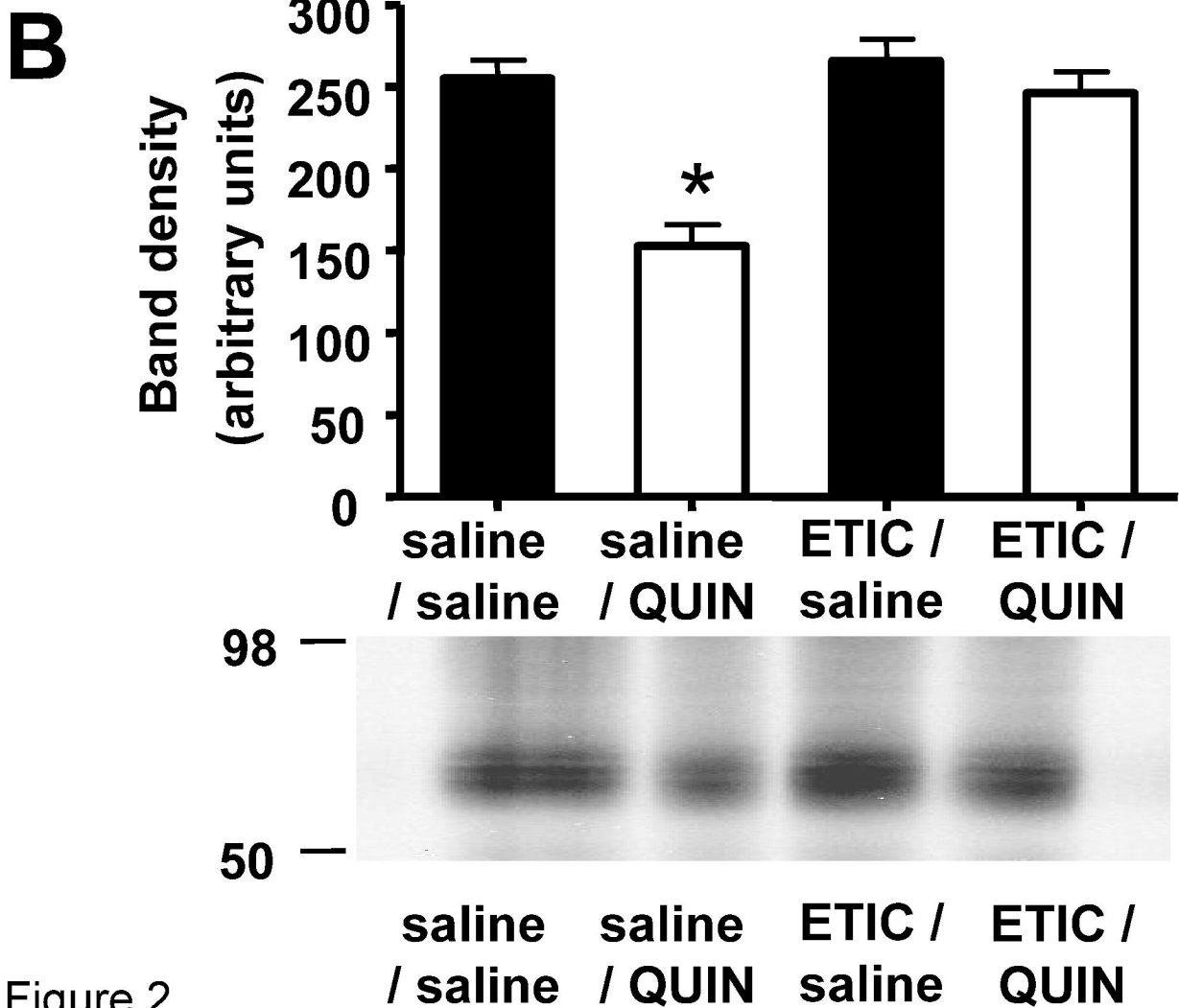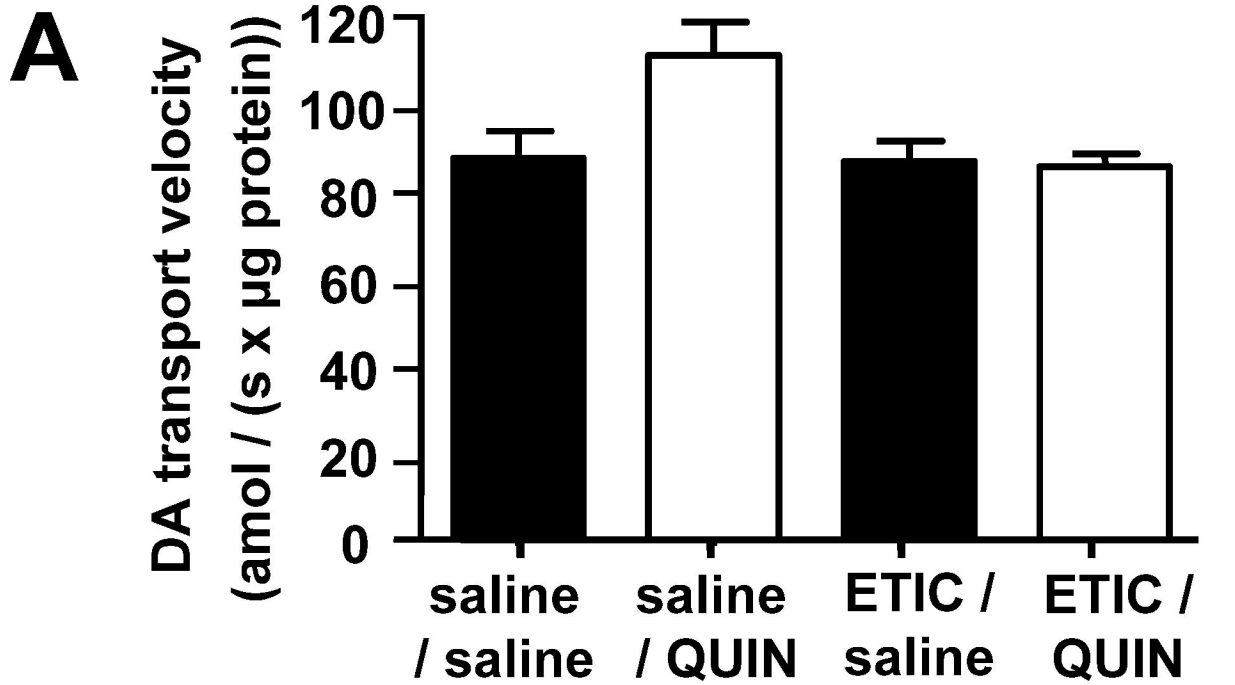Figure 2

membrane-associated vesicles   **cytoplasmic vesicles**

*

saline

MPD

*

DA content (ng / g wet weight)

50

40

30

20

10

0

saline   MPD   saline   MPD

Figure 3

membrane-associated vesicles　　**cytoplasmic vesicles**

■ saline
□ QUIN

DA content (ng / g wet weight)

150

120

90

60

30

0

saline　QUIN　saline　QUIN

*　*

Figure 4

A

DA release velocity
(nmol / (s x g wet weight))

*

saline
/ saline   saline
/ MPD   ETIC /
saline   ETIC
/ MPD

B

DA release magnitude
(nmol / g wet weight)

*

saline
/ saline   saline
/ MPD   ETIC /
saline   ETIC
/ MPD

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9