Regulation of the Vesicular Monoamine Transporter-2: A Novel Mechanism for Cocaine and Other Psychostimulants

JEFFREY M. BROWN, GLEN R. HANSON, and ANNETTE E. FLECKENSTEIN
Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, Utah

ABSTRACT
The plasmalemmal dopamine (DA) transporter (DAT) is a principal site of action for cocaine. This report presents the novel finding that in addition to inhibiting DAT function, cocaine administration rapidly alters vesicular DA transport. Specifically, cocaine treatment abruptly and reversibly increased both the \( V_{\text{max}} \) of DA uptake and the \( B_{\text{max}} \) of vesicular monoamine transporter-2 (VMAT-2) ligand (dihydrotetrabenazine) binding, as assessed ex vivo in purified rat striatal synaptic vesicles. Selective inhibitors of the DAT (amfonelic acid and GBR12935), but not the plasmalemmal serotonin transporter (fluoxetine), also increased vesicular DA uptake. Moreover, DA depletion resulting from administration of the tyrosine hydroxylase inhibitor \( \alpha \)-methyl-\( p \)-tyrosine had cocaine-like effects. Conversely, administration of the DA-releasing agent methamphetamine rapidly decreased vesicular uptake. Taken together, these data demonstrate for the first time ex vivo that cocaine treatment rapidly alters vesicular monoamine transport, and suggest that alterations in cytoplasmic DA concentrations contribute to stimulant-induced changes in vesicular DA uptake. Hence, the VMAT-2 may be an important target for developing strategies to treat not only cocaine addiction but also other disorders involving alterations in neuronal DA disposition, including Parkinson’s disease.

Cocaine is an important psychostimulant of abuse that exerts its addictive and psychomotor effects by elevating extracellular concentrations of dopamine (DA) (Wilson and Schuster, 1972; Roberts and Koob, 1982; Ritz et al., 1987). It is generally accepted that cocaine-induced increases in extracellular DA levels are due principally to the ability of this drug to inhibit the plasmalemmal DA transporter (DAT). Although it has been demonstrated that cocaine-mediated increases in extraneuronal DA are dependent on the existence of a vesicular pool of this catecholamine (Hurd and Ungerstedt, 1989; Pfifl et al., 1995), the precise contribution of the DA-containing vesicles to the effects of this stimulant is unclear.

The vesicular monoamine transporter-2 [VMAT-2; formerly referred to as the SVAT or MAT (Erickson et al., 1992)] is the neuronal element solely responsible for transporting cytoplasmic DA into vesicles for storage and subsequent release in the central nervous system (for review, see Schuldiner, 1994). This transporter is often considered resistant to regulation, because studies assessing persistent effects of drug treatments have demonstrated alterations in DAT and \( D_2 \) DA receptors without concurrent effects on binding of the VMAT-2 ligand methoxytetrabenazine (Vander Borght et al., 1995) or dihydrotetrabenazine (DHTBZ) (Wilson and Kish, 1996). However, recent studies have demonstrated that vesicular DA uptake is decreased 1 h (Brown et al., 2000) and 24 h (Brown et al., 2000; Hogan et al., 2000) after administration of the psychostimulant methamphetamine (METH), as assessed ex vivo in vesicles purified from the striatum of treated rats. Hence, the purpose of this study was to determine whether cocaine administration, like METH, rapidly alters vesicular DA uptake. The results of this study demonstrate that vesicular monoamine transport can be rapidly altered by administration of several distinct dopaminergic agents other than METH, and demonstrate a heretofore unreported mechanism whereby cocaine alters dopaminergic neuronal function.

Materials and Methods

Animals. Male Sprague-Dawley rats (280–330 g; Simonsen Laboratories, Gilroy, CA) were maintained under controlled light and temperature conditions, with food and water provided ad libitum. Rats were sacrificed by decapitation. All experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

Drugs and Radioligands. (±)-Methamphetamine and (-)-cocaine hydrochloride were supplied by the National Institute on Drug Abuse (Rockville, MD). Amfonelic acid and GBR12935 were purchased from Research Biochemicals International (Natick, MA). Flu-
oxetine was obtained from Eli Lilly (Indianapolis, IN). α-Methyl-μ-tyrosine was purchased from Sigma Chemical Co. (St. Louis, MO). 7,8-[3H]DA (49 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL) and α-[2-3H]DHTBZ (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Tetrabenazine was kindly donated by Drs. Jeffrey Erickson, Helene Varoqui (Louisiana State University Health Sciences Center, New Orleans, LA), and Erik Floor (University of Kansas, Lawrence, KS). Drugs were administered as indicated in figure legends; doses were calculated as the respective free base.

Preparation of Rat Striatal Synaptic Vesicles. Synaptic vesicles were obtained from synaptosomes prepared from rat striatum as described previously (Fleckenstein et al., 1997). Synaptosomes were resuspended and homogenized in cold distilled deionized water. Osmolarity was restored by addition of HEPES and potassium tartrate 25 and 100 mM (final concentrations; pH 7.5), respectively. Samples were centrifuged for 20 min at 20,000 g (4°C) to remove lysed synaptosomal membranes. MgSO4 (1 mM, final concentration) was added to the supernatant, which was then centrifuged for 45 min at 100,000 g (4°C). The resulting vesicular pellet was resuspended in wash buffer (see below) at a concentration of 50 mg/ml (original tissue wet weight). Based on published reports using similar protocols for vesicle preparation (Kadota and Kadota, 1973; Teng et al., 1997) we believe vesicles isolated in these studies to be of the small synaptic vesicle size (∼50 nm), the predominant type found in dopaminergic terminals in the striatum (Nirenberg et al., 1997).

Vesicular [3H]DA Uptake and [3H]DHTBZ Binding. Vesicular [3H]DA uptake was performed by incubating 100 μl of synaptic vesicle samples (∼2.5 μg of protein) at 30°C for 3 min in assay buffer (final concentration: 25 mM HEPES, 100 mM potassium tartrate, 1.7 mM ascorbic acid, 0.05 mM EGTA, 0.1 mM EDTA, 2 mM ATP-Mg2+, pH 7.5) in the presence of [3H]DA (30 nM final concentration except in kinetic analyses wherein 0.8−10 μM DA was used). The reaction was terminated by addition of 1 ml of ice-cold wash buffer (assay buffer containing 2 mM MgSO4 substituted for the ATP-Mg2+, pH 7.5) and rapid filtration through Whatman GF/F filters soaked previously in 0.5% polyethylenimine. Filters were washed three times with ice-cold wash buffer using a Brandel filtering manifold. Radioactivity trapped in filters was counted using a liquid scintillation counter. Nonspecific values were determined by measuring vesicular [3H]DA uptake at 4°C in wash buffer.

Binding of DHTBZ was performed as described by Teng et al. (1998). Briefly, 200 μl of the synaptic vesicle preparation (∼6 μg of protein) was incubated in wash buffer in the presence of [3H]DHTBZ (2 nM final concentration except in kinetic analyses wherein 0.25−500 nM DHTBZ was used) for 10 min at 25°C. The reaction was terminated by addition of 4 ml of ice-cold wash buffer and rapid filtration through Whatman GF/F filters soaked in 0.5% polyethylenimine. Filters were washed three times with ice-cold wash buffer. Nonspecific binding was determined by coincubation with 20 μM tetrabenazine. All protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad, Richmond, CA).

DA Determination. Striatal DA content was determined essentially as described by Chapin et al. (1986). Frozen striatal tissue was thawed and sonicated for 6 s in tissue buffer [0.1 M phosphate-citrate buffer (pH 2.5) containing 15% methanol]. Samples were then centrifuged for 5 min at 22,000g. Tissue pellets were retained and protein determined according to the method of Lowry et al. (1951). The supernatant was then injected onto a Partisphere C18 reverse-phase analytical column (5-μm spheres; 110 × 4.6 mm, equipped with a reverse-phase guard column (Whatman Inc., Clifton, NJ). The mobile phase consisted of 0.05 M sodium phosphate, 0.03 M citrate buffer (pH 2.5) containing 0.1 M EDTA, 0.35% sodium octylsulfate, and 25% methanol. DA was detected with an amperometric electrode detector with the working electrode potential set at +0.73 V relative to a Ag+/AgCl reference electrode.

Statistical Analyses. Statistical analyses were performed using an ANOVA followed by a Fisher’s protected least-significant difference post hoc comparison or Student’s t test as indicated. Differences were considered significant if probability of error was ≤0.05.

Results

Results presented in Fig. 1 demonstrate that a single administration of cocaine rapidly increased vesicular [3H]DA uptake, as assessed by measuring [3H]DA uptake into purified striatal vesicles prepared from saline- and cocaine-treated rats. This increase was dose-dependent (Fig. 2), with doses of 15 and 30 mg/kg (i.p.) [doses comparable to produce a maximal increase in cocaine-induced locomotor activity (Bedford et al., 1980)] effecting the greatest increase in vesicular [3H]DA uptake. Multiple administrations of cocaine (four injections, 2-h intervals; 30 mg/kg i.p.) increased vesicular [3H]DA uptake as well (Fig. 1). These increases in vesicular uptake were principally associated with VMAT-2 found in DA and not serotonin neurons, because the majority of striatal VMAT-2 is located in DA neurons (Darchen et al., 1989; Brown et al., 2000). These effects did not result from residual cocaine introduced by the in vivo treatment, because direct application of cocaine at concentrations of 0.01 to 10 μM was without effect, and greater concentrations decreased vesicular uptake in this preparation. (The IC50 for cocaine decreasing vesicular DA uptake was 135 ± 9 μM; a value similar to that reported previously; Reith et al., 1994.) DHTBZ binding, but not affinity, was also increased after both a single and multiple cocaine injections (in nM and fmol/μg of protein: Kd and Bmax = 8.5 and 28 after saline treatment versus 8.6 and 36 after a single 30 mg/kg i.p. cocaine injection; Kd and Bmax = 10 and 28 after saline treatment versus 10 and 37 after 4 × 30-mg/kg i.p. injections of cocaine; 2-h intervals). Interestingly, this increase in DHTBZ binding was only apparent when assessing DHTBZ binding in purified vesicular preparations: no difference in DHTBZ binding was detected when assays were performed in striatal tissue homogenates prepared from saline- and cocaine-treated rats (data not shown). In addition, neither METH nor cocaine treatment seemed to affect the yield of synaptic vesicles in the preparation, as evidence by findings that total protein concentrations in the vesicular fraction were not altered by drug treatment (data not shown).
The enhancement of vesicular DA uptake after both single and multiple cocaine administrations appeared similar, because both treatments increased $V_{\text{max}}$ and not $K_{\text{m}}$ of uptake (in pmol/mg/min and nM: 296 and 221 after saline treatment versus 381 and 224 after a single 30 mg/kg cocaine injection; 188 and 269 after saline treatment versus 255 and 312 after 30-mg/kg i.p. injections of cocaine; 2-h intervals). Consequently, effects of a single cocaine treatment were characterized. Results presented in Fig. 3 demonstrate that the increase in vesicular uptake after a single 30-mg/kg injection occurred by 30 min after treatment, but subsided by 6 h.

The ability of cocaine to directly inhibit DAT is well established (Heikkila et al., 1975; Ritz et al., 1987; Nicolaysen and Justice, 1988; Kilty et al., 1991; Shimada et al., 1991); hence, the hypothesis that blockade of DAT contributed to the cocaine-induced increase in vesicular uptake was explored. Results presented in Fig. 4 suggest an interaction between these proteins, because a single administration of two selective inhibitors of DAT, amfonelic acid or GBR12935, rapidly increased vesicular DA uptake in a cocaine-like manner. This phenomenon was selective for DAT inhibitors as evidenced by the finding that administration of the plasmalemmal serotonin transporter inhibitor fluoxetine was without effect on vesicular DA uptake (Fig. 4).

Although the effects of DAT inhibitors on cytoplasmic DA concentrations have not been reported, we hypothesize that prevention of DA reuptake with cocaine, amfonelic acid, or GBR12935 transiently decreases cytoplasmic DA concentrations. To determine whether such a decrease provides a mechanism linking the activities of DAT and VMAT-2, effects of DA depletion on vesicular DA uptake were determined. DA was depleted by administering the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (αMPT; Moore and Dominic, 1971): this treatment decreased striatal DA concentrations by 45% (i.e., from 165.2–91.2 ng/mg of protein). Results presented in Fig. 5 confirm that similar to effects of cocaine treatment, αMPT increased vesicular $[3H]$DA uptake. The magnitude of DA depletion by αMPT was inversely correlated with the degree of increase in vesicular uptake when comparing individual animals ($R^2 = 0.335$, $p < 0.01$, $n = 18$).

To test further the hypothesis that alterations in DA disposition might regulate the activity of VMAT-2, the effects of METH were assessed. It is established that METH causes nonvesicular release of DA via the DAT. As recently reported (Brown et al., 2000), multiple METH injections (four injections, 2-h intervals; 10 mg/kg s.c.) decreased vesicular $[3H]$DA uptake within 1 h after administration (Fig. 6). A single METH injection (15 mg/kg s.c.) also decreased vesicular $[3H]$DA uptake within 1 h, albeit to a lesser extent (Fig. 6). Similar to the effects of cocaine, this rapid phenomenon was not attributable to residual drug introduced by the original METH treatment (Brown et al., 2000).

---

**Fig. 2.** Rats received a single injection of cocaine (5–30 mg/kg i.p.) or saline vehicle (1 ml/kg i.p.) and were decapitated 1 h later. Columns represent the means and vertical lines 1 S.E.M. of determinations in six rats. *Values for cocaine-treated rats that are significantly different from saline-treated controls ($p \leq 0.05$).

**Fig. 3.** Rats received a single injection of cocaine (30 mg/kg i.p.) or saline vehicle (1 ml/kg i.p.) and were decapitated 15 min to 6 h later. Symbols represent the means and vertical lines 1 S.E.M. of determinations in six rats. Data are expressed as a percentage of the mean of control. Mean control values for $[3H]$DA uptake and $[3H]$DHTBZ binding ranged from 67.8 to 176.5 fmol/µg and 1.4 to 2.3 fmol/µg, respectively. *Values for cocaine-treated rats that are significantly different from saline-treated controls ($p \leq 0.05$).

**Fig. 4.** Rats received a single administration of cocaine (30 mg/kg i.p.), amfonelic acid (AA; 10 mg/kg i.p.), GBR-12935 (GBR; 20 mg/kg i.p.), fluoxetine (Fluox; 10 mg/kg i.p.), or saline vehicle (1 ml/kg i.p.) and were decapitated 1 h later. Columns represent the means and vertical lines 1 S.E.M. of determinations in six rats. Data are expressed as a percentage of the mean of control. The control values for $[3H]$DA uptake and $[3H]$DHTBZ binding ranged from 159.3 to 199.5 fmol/µg and 3.6 to 5.2 fmol/µg, respectively. *Values for cocaine-treated rats that are significantly different from saline-treated controls ($p \leq 0.05$).
Discussion

It is accepted widely that the principal action of cocaine is to inhibit plasmalemmal monoamine transporter function. Furthermore, it has been suggested that VMAT-2, unlike DAT, is resistant to regulation. For instance, it was reported that chronic cocaine self-administration alters rat striatal and nucleus accumbens DAT levels, as estimated by measuring $[^{3}H]$WIN35,428 and $[^{3}H]$GBR12935 binding (Wilson et al., 1994), but is without effect on $[^{3}H]$DHTBZ binding (Wilson and Kish, 1996). In contrast, the data presented herein demonstrate that cocaine induces a rapid and reversible increase in vesicular dopamine uptake and binding. Although the significance of enhanced vesicular uptake remains to be determined, it is possible that cocaine, by inhibition of the DAT and increasing vesicular sequestration of DA, causes a shift in the ratio of cytoplasmic to vesicular DA such that less is retained in the cytoplasm and more is packaged in each vesicle before its release. Interestingly, Pothis et al. (2000) recently suggested that regulation of vesicular transporter activity may cause rapid and profound alterations in neurotransmitter release. Accordingly, a “DA-releasing” action of cocaine has been suggested previously (Hurd and Ungerstedt, 1989; Pifl et al., 1995), and may represent a significant mechanism whereby cocaine increases extraneuronal DA concentrations. Hence, the VMAT-2 may represent a novel target for developing agents useful for treating cocaine abusers.

The discrepancy between the present finding that cocaine alters vesicular dopamine transport and findings by others that the VMAT-2 is not altered by treatment with dopaminergic agents may be due to several factors. For instance, in the studies by Wilson and Kish (1996), rats were exposed to cocaine for 3 weeks and VMAT-2 binding was assessed on the last day or 3 weeks after treatment. In contrast, vesicular binding and uptake were assessed in the present study 1 h after a single or subchronic cocaine treatment regimen. Hence, it is possible that tolerance to the rapid VMAT-2 effect reported herein may have occurred during the chronic treatment regimen used by Wilson and Kish (1996). Alternatively, data presented in Fig. 3 demonstrate that this cocaine-induced increase in vesicular $[^{3}H]$DA uptake and $[^{3}H]$DHTBZ binding subsides by 6 h and therefore would not have been detected by Wilson and Kish (1996) who assessed effects 1 day and 3 weeks after treatment. Another interesting possibility stems from the fact that studies assessing VMAT-2 changes are typically conducted using whole brain sections, whereas the rapid effect reported herein was apparent only in purified synaptic vesicles. In fact, overall DHTBZ binding was not significantly affected by cocaine administration in the present studies when assessed in whole striatal homogenate preparations. A similar discordance between DHTBZ binding in homogenate and purified vesicular preparations was reported recently by Hogan et al. (2000), who observed no change in homogenate, but a decrease in VMAT-2 binding, in purified vesicular preparations obtained 24 h after multiple METH administrations. These data suggest that the rapid effect on vesicular dopamine uptake reported herein occurs in the synaptic vesicles purified in the present study, and is not detected when assessing DHTBZ binding in homogenates or whole slices. This fact may be due to the presence of VMAT-2 on large dense core vesicles and tubulovesicular organelles that may be eliminated upon purification of small synaptic vesicles (under Materials and Methods). Alternatively, these findings may be explained by a redistribution of vesicles within the neuron, which may not be detected in a slice or homogenate preparation.

It is well established that cocaine inhibits DAT function. Since the VMAT-2 in our purified synaptic vesicle preparation was principally associated with DAT-containing neurons, a causal relationship between the decrease in DAT and increase in VMAT-2 activities was suggested. Accordingly, the effects of other plasmalemmal uptake inhibitors were assessed. It was determined that GBR12935 and amfonelic acid, like cocaine, comparably increased vesicular DA uptake, suggesting an interaction between DAT and VMAT-2. To investigate further this association, effects of DA depletion were assessed since we hypothesized that by preventing DA reuptake, cocaine, amfonelic acid, or GBR12935 may transiently decrease cytoplasmic DA concentrations. Results presented in Fig. 5 support this hypothesis by demonstrating...
what, similar to the effects of cocaine treatment, depletion of DA by αMPT-treatment increased vesicular [3H]DA uptake.

Results presented in Fig. 6 demonstrate that in contrast to the effects of cocaine, multiple METH administrations rapidly and profoundly decrease VMAT-2 function. Although the functional significance of this decrease has yet to be determined, it is possible that this decrement causes a shift in the ratio of cytoplasmic to vesicular DA such that more is retained in the cytoplasm and less in each vesicle. Interestingly, Sulzer et al. (1995) have demonstrated that the METH analog amphetamine reduces quantal DA release from PC12 cells by greater than 50% per vesicle. These authors speculated that this decrease may be related to either a collapse of the proton gradient that provides free energy for DA packaging or a blockade of VMAT-2; the present data are consistent with the latter.

The hypothesis that increases in intraneuronal DA concentrations decrease the activity of VMAT-2 is supported by a recent report by Lee et al. (1999) comparing vesicular uptake in fibroblasts modified to express VMAT-2 with cells coexpressing the DA-synthesizing enzyme aromatic amino acid decarboxylase (AADC) and VMAT-2. In this study, fibroblasts containing only VMAT had lower intracellular dopamine levels compared with cells expressing both VMAT and AADC. However, fibroblasts with higher intracellular dopamine levels (AADC- and VMAT-expressing fibroblasts) had lower VMAT activity, thereby supporting the assertion that increases in cytoplasmic DA decrease VMAT-2 activity.

Data presented in Figs. 1 to 6 provide some of the first ex vivo evidence that psychostimulants such as cocaine and METH have profound and rapid effects on vesicular uptake. This phenomenon likely contributes significantly to DA responses to a wide variety of pharmacological treatments. For instance, differences in vesicular uptake after METH and cocaine administration may underlie the dissimilar neurotoxic profile of these stimulants. Specifically, METH, but not cocaine, causes persistent DA deficits presumably associated with nerve terminal degeneration (Hotchkiss et al., 1979; Ricauer et al., 1982, 1984; Gibb et al., 1990). It has been suggested that METH causes DA terminal loss by effecting an accumulation of DA in the cytoplasm that, in turn, causes formation of neurotoxic reactive oxygen species (Cubells et al., 1994). A decrease in vesicular uptake following METH treatment, such as that depicted in Fig. 6, might contribute to an increase in intracellular DA levels and thereby cause long-term damage. Interestingly, a role for VMAT-2 in effecting the DA neurotoxicity caused by METH treatment has been suggested by findings of enhanced METH-induced dopaminergic deficits in heterozygotic transgenic mice lacking 50% of their VMAT-2 (Fumagalli et al., 1999). In contrast to the effects of METH, cocaine does not increase cytosolic DA levels, both because it prevents the reuptake of newly released DA and because increased vesicular uptake would likely lower cytosolic DA concentrations. Hence, cocaine is predictably not neurotoxic. Interestingly, it has been demonstrated that the cocaine-like agent amfonelic acid, administered 8 h after a neurotoxic METH treatment, prevents the persistent DA deficits caused by the stimulant (Marek et al., 1990): this may be due to a cocaine-induced enhancement of vesicular uptake that promotes sequestration of the elevated intraneuronal DA caused by METH treatment.

Pharmacologically altering vesicular DA uptake has important implications beyond explaining differences between the long-term effects of METH and cocaine. For instance, it has been suggested that DA neurons with reduced VMAT-2 expression may be more susceptible to damage caused by autoxidation of cytoplasmic DA (Miller et al., 1999), presumably because less is sequestered within vesicles and therefore available to promote reactive oxygen species formation. Autoxidation of cytoplasmic DA is thought to contribute to development of Parkinson’s disease. Accordingly, pharmacological manipulations that increase vesicular uptake such as the DAT inhibitors may be useful in slowing the progression of Parkinson’s disease by stimulating vesicular removal of DA from potentially unstable cytoplasmic DA pools. Precedence for a neuroprotective sequestering function for VMAT-2 in a Parkinsonian model was established previously by the demonstration that VMAT-2 sequesters and thereby protects against the DA neuronal damage caused by the neurotoxin 1-methyl-4-phenylpyridinium (MPP+). Lui et al., (1992). Whether a pharmacological enhancement of VMAT-2 function would have clinical relevance remains to be established. However, data presented here suggest that vesicular DA uptake can be regulated and may be a valuable target for treatment of not only cocaine addiction but also other disorders involving disruption of normal DA disposition.

References
Marek GJ, Vosmer G and Seiden LS (1990) Dopamine uptake inhibitors block...
long-term neurotoxic effects of methamphetamine upon dopaminergic neurons.


Send reprint requests to: Annette E. Fleckenstein, Ph.D., University of Utah, Department of Pharmacology and Toxicology, 30 South 2000 East Rm. 201, Salt Lake City, UT 84112. E-mail: fleckenstein@hsc.utah.edu