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The molecular mechanism of "ecstasy" [3,4-methylenedioxymethamphetamine (MDMA)]: Serotonin transporters are targets for MDMA-induced serotonin release

GARY RUDNICK* AND STEPHEN C. WALL

Department of Pharmacology, Yale University School of Medicine, P.O. Box 3333, New Haven, CT 06510

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ABSTRACT **MDMA ("ecstasy") has been widely reported as a drug of abuse and as a neurotoxin. This report describes the mechanism of MDMA action at serotonin transporters from plasma membranes and secretory vesicles. MDMA stimulates serotonin efflux from both types of membrane vesicle. In plasma membrane vesicles isolated from human platelets, MDMA inhibits serotonin transport and [^{3}H]imipramine binding by direct interaction with the Na^+-dependent serotonin transporter. MDMA stimulates radiolabel efflux from plasma membrane vesicles preloaded with [^{3}H]serotonin in a stereospecific, Na^+-dependent, and imipramine-sensitive manner characteristic of transporter-mediated exchange. In membrane vesicles isolated from bovine adrenal chromaffin granules, which contain the vesicular biogenic amine transporter, MDMA inhibits ATP-dependent [^{3}H]serotonin accumulation and stimulates efflux of previously accumulated [^{3}H]serotonin. Stimulation of vesicular [^{3}H]serotonin efflux is due to dissipation of the transmembrane pH difference generated by ATP hydrolysis and to direct interaction with the vesicular amine transporter.**

Serotonin transport has been implicated in the mechanism of a number of amphetamine derivatives including *p*-chloroamphetamine, fenfluramine, 3,4-methylenedioxyamphetamine, and MDMA. These compounds cause an acute release of serotonin *in vivo* (1, 2) and *in vitro* (3–5) and also lead to a long-term depletion of serotonin (6, 7) that correlates with morphological damage to serotonergic nerve endings (7–10). The serotonin transporter has been implicated in these phenomena since inhibitors of serotonin transport block the effect of amphetamine derivatives on acute serotonin release and destruction of serotonergic terminals (5, 11, 12). These results suggest that the serotonin transporter either mediates the entry of neurotoxic amphetamines into serotonergic terminals or participates in sequelae leading to serotonin release and depletion (13) or both.

The observation that Ca^{2+} is not required for amphetamine-induced serotonin release (4) suggests that exocytosis is not involved. Thus, it is likely that amphetamines may induce release by reversal of the transport systems that normally catalyze accumulation of serotonin to high levels within the neuron and the synaptic vesicle. We, therefore, examined two membrane vesicle model systems for serotonin transport to determine if MDMA directly affects these transport systems. Serotonergic neurons, like other cells that secrete serotonin, contain two serotonin transport systems that function in series (14). One of these systems transports serotonin into the cell, and the other sequesters intracellular serotonin within secretory vesicles.

Purified platelet plasma membrane vesicles contain the Na^+-dependent imipramine-sensitive serotonin transporter responsible for serotonin reuptake into presynaptic nerve endings (15). When appropriate transmembrane ion gradients are imposed, these vesicles accumulate [^{3}H]serotonin to concentrations several hundredfold higher than in the external medium. Transport requires external Na^+ and Cl^- and is stimulated by internal K^+ (16–18). Results from a variety of experiments suggest that serotonin is transported across the membrane with Na^+ and Cl^- in one step of the reaction and that K^+ is transported in the opposite direction in a second step (19).

Membrane vesicles isolated from bovine adrenal chromaffin granules accumulate biogenic amines, including serotonin, by an H^+-coupled transport system driven by ATP hydrolysis (20). Apparently, the same system is responsible for amine uptake into secretory vesicles from platelets, mast cells, and nerve endings (21–23). The system consists of two components: (*i*) an ATP-driven H^+ pump, the vacuolar ATPase, which acidifies the vesicle interior and also generates a transmembrane electrical potential ($\Delta\psi$, interior positive) (24), and (*ii*) the reserpine-sensitive vesicular amine transporter, which couples efflux of one or more H^+ ions to the uptake of each molecule of biogenic amine (25).

Previous work (3–5) *in vitro* using MDMA has been performed with synaptosomes or brain slices. Those studies did not distinguish between effects on the plasma membrane and vesicular transporters. In the present work, we have examined the interaction of MDMA with each of these transporters separately. The results suggest that MDMA is a substrate for both transport systems and imply that MDMA causes serotonin release by stimulating serotonin–MDMA exchange.

EXPERIMENTAL PROCEDURES

Membrane Vesicles. Outdated human platelet concentrates were purchased from the Connecticut Red Cross. Platelets from 50 to 100 individuals were pooled for each membrane preparation. Platelet plasma membrane vesicles were isolated by the method of Barber and Jamieson (26) with the modifications described (27). Chromaffin granule membrane vesicles were prepared as described by Schuldiner *et al.* (28) by repeated osmotic lysis of bovine adrenal medullary chromaffin granules isolated by differential sedimentation.

Transport Assays. Transport of serotonin into plasma membrane vesicles was measured at 25°C using the filtration assay (15). Unless otherwise indicated, vesicles were equilibrated with 10 mM lithium phosphate (pH 6.7) containing 133 mM K_2SO_4 and 1 mM $MgSO_4$ and diluted into external medium containing 0.2 M NaCl, 10 mM lithium phosphate (pH 6.7), 1 mM $MgSO_4$, and a subsaturating concentration (0.1 μM) of [^{3}H]serotonin (12.3 Ci/mmol; 1 Ci = 37 GBq). Chromaffin granule membrane vesicles were diluted to a concentration of ≈0.3 mg/ml in (unless indicated otherwise) 0.3 M sucrose

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Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; ΔpH, transmembrane pH difference.

containing 10 mM Hepes (adjusted to pH 8.5 with KOH), 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM disodium ATP, and 0.1 μM [^{3}H]serotonin, which is below the K_m for transport. Reactions (200 μl per assay) were stopped by dilution, filtration, and washing, and radioactivity in filtered vesicles was measured as described (28). The standard error of replicate assay values was typically <5% of the mean.

Serotonin Efflux and Exchange. Platelet plasma membrane vesicles equilibrated with 10 mM lithium phosphate (pH 6.7) containing 60 mM NaCl, 93 mM K_2SO_4, and 1 mM $MgSO_4$ were diluted 1:30 into 0.2 M NaCl containing 10 mM lithium phosphate (pH 6.7) and 0.1 μM [^{3}H]serotonin at 25°C. After serotonin accumulation had reached a maximum (5–10 min), efflux was initiated by a 1:40 dilution of the suspension with the indicated medium. After a 30-sec incubation, the vesicles were collected by filtration through Gelman GN-6 nitrocellulose filters. The filter and reaction tube were rapidly rinsed with 2 ml of ice-cold 0.2 M NaCl, and radioactivity in the filter was measured in 3 ml of Optifluor (Packard). Efflux from chromaffin granule membrane vesicles was measured in the same way, except that preloading was carried out for 40 min in 0.3 M sucrose containing 10 mM Hepes adjusted to pH 8.5 (with KOH), 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM disodium ATP, and 0.1 μM [^{3}H]serotonin and efflux was measured 11 min after dilution into the same medium without ATP, $MgSO_4$, or [^{3}H]serotonin. The standard error of replicate assay values was typically <10% of the mean.

Imipramine Binding. Imipramine binding was measured at 25°C using the filtration assay described (29). Briefly, to initiate binding, membrane vesicles were suspended at a protein concentration of 0.3 mg/ml in an assay buffer of 200 mM NaCl containing 10 mM LiH_2PO_4 (pH 6.7) and 1 mM $MgSO_4$. The assay buffer also contained 0.9 nM [^{3}H]imipramine (48 cpm/fmol), a concentration below the K_d under these conditions. After a 15-min incubation, the reactions (300 μl per assay) were terminated by dilution with 4 ml of ice-cold isoosmotic NaCl and filtered through Whatman GF/B filters pretreated with 0.5% polyethyleneimine. The tube and filter were washed three times with 4 ml of ice-cold NaCl solution. Filters were placed in 3 ml of Optifluor and radioactivity was measured after 5 hr. Binding in the absence of Na^+ or in the presence of 100 μM serotonin was taken as a control for nonspecific binding. The standard error of replicate assay values was typically <5% of the mean.

Transmembrane pH Difference (ΔpH) Measurements. Chromaffin granule membrane vesicles (80 μg of protein) were incubated at room temperature in a cuvette containing 2 ml of 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid (Epps) (adjusted to pH 8.5 with KOH), 150 mM KCl, 6 μM acridine orange, 0.2 mM EDTA, and 5 mM ATP. The relative fluorescence of the mixture was measured using an excitation wavelength of 490 nm and an emission wavelength of 526 nm on a Perkin–Elmer LS-5B luminescence spectrometer. After the baseline fluorescence stabilized, acidification was initiated by the addition of 6 mM $MgSO_4$, and the relative fluorescence was monitored. After there was no further detectable quenching (≈20 min), MDMA was added and the increase in fluorescence was measured. This procedure was repeated several times, and after the last addition, 20 mM NH_4Cl was added to neutralize the vesicle interior and completely reverse the quenching. In replicate experiments, the MDMA concentration required for half-maximal reversal of the fluorescence quenching varied by <10% of the mean value.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (30) using bovine serum albumin as a standard.

Materials. [^{3}H]Serotonin (12.3 Ci/mmol) and [^{3}H]imipramine (40.4 Ci/mmol) were purchased from Amersham and New England Nuclear, respectively. Stereoisomers of MDMA were obtained from the National Institute on Drug Abuse. All other reagents were reagent grade, purchased from commercial sources.

RESULTS

Plasma Membrane Vesicles. MDMA is a potent inhibitor of serotonin transport into plasma membrane vesicles. As shown in Fig. 1 (solid lines), the initial rate of [^{3}H]serotonin uptake is inhibited by >60% at 1 μM (+)-MDMA and is inhibited by >97% at 10 μM MDMA. The (−)-isomer is less potent, with <10% inhibition at 1 μM MDMA. Although this inhibition is likely to represent competition at the transport site, it could also result from nonspecific dissipation of the ion gradients that drive transport, for example, by permeabilization of the membrane. Fig. 1 (dotted lines) shows, however, that MDMA interacts directly with the serotonin transporter by virtue of its ability to displace imipramine. In this experiment, equilibrium binding of [^{3}H]imipramine was inhibited 50% by ≈20 μM (+)-MDMA and >90% by 300 μM (+)-MDMA. Again, the (−)-isomer was less potent. In separate experiments (not shown), the reversible nature of MDMA inhibition was demonstrated. Transport into vesicles was inhibited 85% by incubation with 3 μM MDMA, but sedimenting and resuspending the vesicles in fresh buffer completely reversed the inhibition. Inhibition of transport by (+)-MDMA was competitive with serotonin with a K_i of 0.31 ± 0.02 μM (data not shown).

To test for the ability of MDMA to exchange directly with serotonin, we allowed membrane vesicles to accumulate [^{3}H]serotonin and then diluted them 1:40 into medium free of [^{3}H]serotonin. As shown in Fig. 2, rapid loss of radiolabel occurs from these vesicles, which contain internal NaCl, when the dilution medium contains 30 mM K^+. This efflux represents reversal of the normal transport reaction. Removal of K^+ from the dilution medium inhibits efflux by decreasing the rate of transporter reorientation (31), which becomes rate-determining under these conditions. Addition of 1 μM unlabeled serotonin in the absence of external K^+ increases efflux by a process of exchange, which bypasses the slow step of transporter reorientation (31). Addition of 10 μM (+)-MDMA stimulates loss of radiolabel even more than serotonin, suggesting that MDMA is a substrate for the transporter and can replace serotonin in the transport reaction.

According to the previously proposed mechanism for serotonin transport (31), each time the transporter releases a molecule of [^{3}H]serotonin to the vesicle exterior, it must undergo a slow conformational reorientation before binding another molecule of internal [^{3}H]serotonin. This slow step is accelerated directly by external K^+, which is concomitantly transported in. Binding of unlabeled serotonin (or, apparently, MDMA) bypasses the slow step by allowing reversal of the relatively fast substrate transport step. In support of this role for MDMA, the concentration dependence for MDMA stimulation of serotonin efflux (Fig. 1, dashed lines) is similar to that for its inhibition of serotonin influx. Furthermore, the stereospecificity observed for inhibition of transport and binding is maintained for MDMA-induced efflux, with the (+)-isomer being more potent (Fig. 1).

The results in Table 1 argue against the possibility that efflux stimulation results from nonspecific effects, such as increasing the permeability of the membrane to serotonin. The control treatment shows the effect of serotonin and MDMA on increasing [^{3}H]serotonin efflux. Previous work suggests that efflux mediated by the serotonin transporter requires internal Na^+ and Cl^- as cotransported ions (17, 31). Vesicles in which internal NaCl is replaced with either LiCl or K_2SO_4 do not lose [^{3}H]serotonin in response to external unlabeled serotonin or MDMA (Table 1). Table 1 shows that

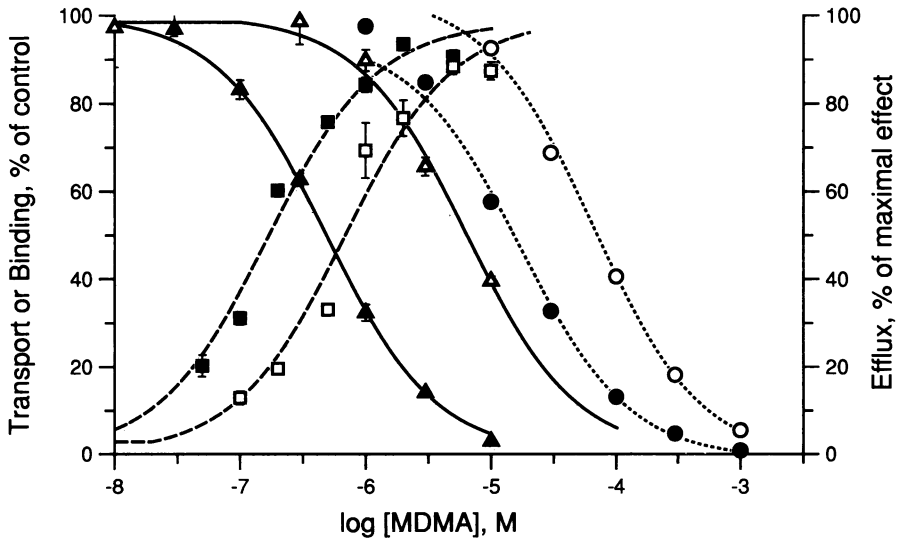

FIG. 1. Effect of MDMA on serotonin transport, imipramine binding, and serotonin efflux by plasma membrane vesicles. Transport is shown by solid lines. Either (+)- or (−)-MDMA (solid or open triangles, respectively) was added at the indicated concentration to the serotonin transport reaction mixture. The control rate of transport in the absence of added MDMA was 120 pmol·mg^{-1}·min^{-1}. Binding is shown by dotted lines. Either (+)- or (−)-MDMA (solid or open circles, respectively) was added at the indicated concentration to the imipramine binding reaction mixture. The control binding under these conditions was 0.52 pmol/mg in the absence of added MDMA. Efflux is shown by dashed lines. Vesicles that had accumulated [^{3}H]serotonin were diluted into NaCl medium containing either (+)- or (−)-MDMA (solid or open squares, respectively) at the indicated concentration, and efflux was measured for 1 min. The maximal extent of efflux was 50% in 1 min at 10 μM MDMA. Error bars represent the standard deviation and are plotted only where they exceeded the size of the symbol.

removal of external Na^+ or Cl^- accelerates efflux, as reported (31). According to the proposed mechanism for exchange (31), stimulation by external substrate should not be observed if external Na^+ or Cl^- is removed. Under these conditions no efflux stimulation is observed with either MDMA or serotonin despite the fact that external K^+ can still increase efflux (see Table 1). Moreover, imipramine, which binds tightly to the serotonin transporter and prevents it from

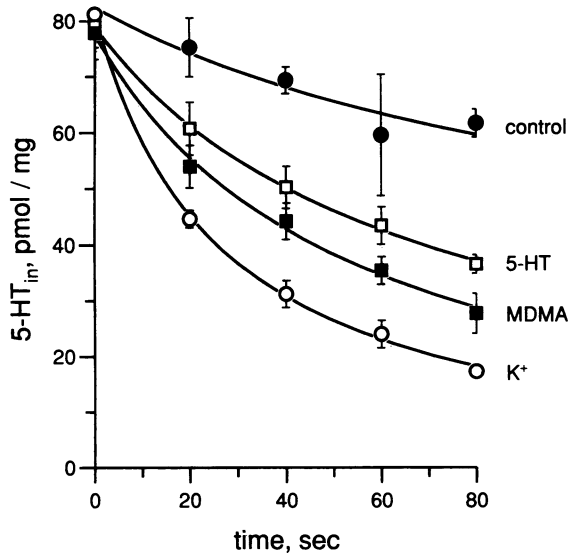

FIG. 2. Time course of [^{3}H]serotonin efflux from plasma membrane vesicles. Vesicles preloaded with [^{3}H]serotonin were diluted into 0.2 M NaCl containing 1 mM $MgSO_4$ and 10 mM lithium phosphate (pH 6.7) (control, solid circles) or the same medium containing 1 μM serotonin (5-HT, open squares) or 10 μM (+)-MDMA (MDMA, solid squares). Alternatively, vesicles were diluted into 0.17 M LiCl containing 30 mM KCl, 1 mM $MgSO_4$, and 10 mM lithium phosphate (pH 6.7, K^+, open circles). At the indicated times, a portion of the diluted vesicle suspension was filtered and washed. Error bars represent the standard deviation and are plotted only where they exceeded the size of the symbol.

catalyzing serotonin translocation, also blocks the ability of MDMA and serotonin to stimulate [^{3}H]serotonin efflux (Table 1).

Storage Vesicles. MDMA also inhibits the ability of chromaffin granule membrane vesicles to accumulate [^{3}H]serotonin. Fig. 3 demonstrates that the initial rate of serotonin influx is inhibited ≈50% at 10 μM (+)-MDMA (solid squares), whereas the (−)-isomer was less potent (open squares). Additional experiments (not shown) demonstrated the competitive nature of this inhibition. A K_i value of 4.72 ± 0.40 μM was calculated for (+)-MDMA in chromaffin granule membrane vesicles. When vesicles preincubated with ATP and [^{3}H]serotonin are diluted 1:40 into medium free of ATP and [^{3}H]serotonin, slow efflux of radiolabel ensues, which is accelerated by (+)-MDMA or NH_4Cl (data not shown). (+)-MDMA is

Table 1. Na^+ dependence and imipramine sensitivity of serotonin–MDMA exchange

Treatment	Efflux, % of internal contents		
	No addition	Serotonin	(+)-MDMA
Control	7.7 ± 4.1	30.0 ± 2.6	35.3 ± 1.8
$LiCl_{in}$	−3.6 ± 2.3	5.5 ± 4.5	6.9 ± 1.7
K_2SO_{4in}	4.2 ± 5.4	9.7 ± 4.2	8.2 ± 3.6
$LiCl_{out}$	28.5 ± 3.0	30.7 ± 2.6	30.5 ± 3.5
Sodium isethionate$_{out}$	24.3 ± 5.1	33.2 ± 4.2	28.5 ± 4.3
Imipramine (2 μM)	11.9 ± 5.3	14.1 ± 5.4	15.7 ± 3.1

Platelet plasma membrane vesicles equilibrated with 60 mM NaCl or with isoosmotic K_2SO_4 were loaded with [^{3}H]serotonin and diluted into the following media: for control and K_2SO_{4in}, 0.2 M NaCl containing 1 mM $MgSO_4$ and 10 mM lithium phosphate (pH 6.7); for $LiCl_{out}$, NaCl was replaced with equimolar LiCl; for imipramine, 2 μM imipramine was added to control medium. Serotonin at 1 μM or (+)-MDMA at 10 μM was added to the dilution medium where indicated. All values represent the percent [^{3}H]serotonin efflux in the first 30 sec after dilution. In the absence of internal NaCl, the vesicles contained 180 pmol of serotonin per mg of membrane protein (≈18 μM) at the time of dilution. In the presence of internal NaCl, this value decreased to 65 pmol/mg or 6.5 μM. When 30 mM K_2SO_4 was present in the dilution medium, the efflux rate rose to 44.6 ± 5.4% of the contents in 30 sec.

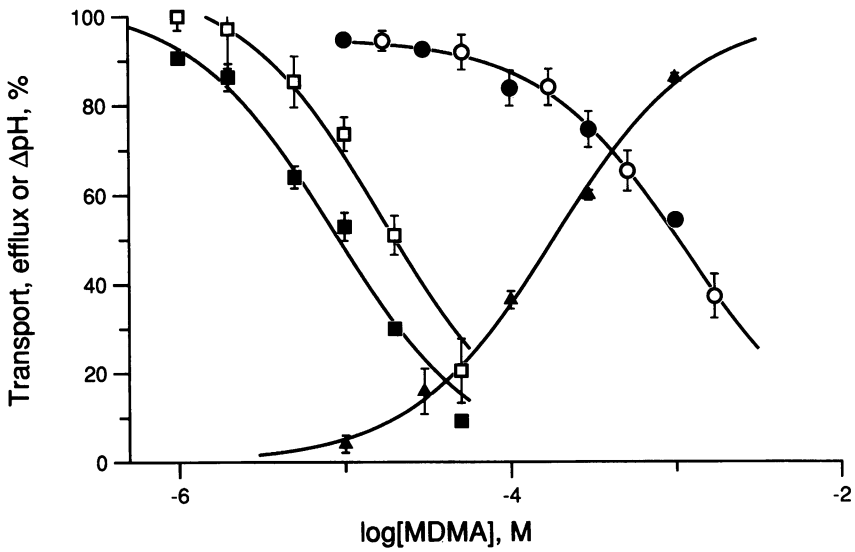

FIG. 3. ΔpH and serotonin flux by chromaffin granule membrane vesicles. For transport, either (+)- or (−)-MDMA (solid or open squares, respectively) was added at the indicated concentration to the serotonin transport reaction mixture. The control rate of transport was 35.7 pmol·mg^{-1}·min^{-1} in the absence of added MDMA. For efflux, vesicles that had accumulated [^{3}H]serotonin were diluted into medium containing (+)-MDMA (triangles) at the indicated concentration and efflux was measured after 11 min. The maximal extent of efflux was 86% in 11 min at 1 mM MDMA. For dissipation of ΔpH, acidification of chromaffin granule membrane vesicles was measured using acridine orange fluorescence. Addition of 20 mM NH_4Cl completely reversed MgATP-dependent quenching. By using the MgATP-dependent and NH_4Cl-sensitive acidification as 100%, the relative effect of (+)- or (−)-MDMA (solid or open circles, respectively) is shown for the various concentrations added. These values represent changes in fluorescence and are not necessarily proportional to ΔpH changes. Error bars represent the standard deviation for transport and efflux or the range for fluorescence quenching and are plotted only where they exceeded the size of the symbol.

more potent than NH_4Cl at stimulating efflux, with a half-maximal activity at <200 μM (Fig. 3, triangles), compared to 1 mM for NH_4Cl. In separate experiments, we tested the stereospecificity of MDMA-induced serotonin efflux. At 300 μM, (+)-MDMA caused 64 ± 2% efflux of serotonin and (−)-MDMA caused 48 ± 4% efflux within the same 11-min period.

Stimulation of serotonin efflux, and inhibition of its uptake, could reflect the ability of MDMA to be transported by the vesicular amine transporter. As a weakly basic amine, however, MDMA can also dissipate, by passive nonionic diffusion, the ΔpH that drives transport. To evaluate these possibilities, we measured the ability of MDMA to dissipate ΔpH as measured by acridine orange fluorescence quenching. The results in Fig. 3 (circles) demonstrate that MDMA dissipates ΔpH with half-maximal effect at ≈1 mM and that each isomer is equally active. Although the assays are different, MDMA appears to be more potent at stimulating efflux than at dissipating ΔpH (Fig. 3). This result and the stereospecificity of MDMA-induced efflux suggest that, although MDMA dissipates ΔpH, it may also exchange with serotonin through the amine transporter.

DISCUSSION

The results presented here using two membrane vesicle model systems demonstrate that MDMA directly and indirectly stimulates serotonin efflux through the plasma membrane and vesicular membrane transport systems responsible for serotonin reuptake and storage in nerve terminals. In plasma membrane vesicles, MDMA-induced efflux is evidently mediated by the serotonin transporter, since the process requires Na^+ on both sides of the membrane and is blocked by imipramine. Direct interaction of MDMA with the transporter is illustrated also by its ability to displace [^{3}H]imipramine in competitive equilibrium binding assays. This observation confirms the finding of Battaglia *et al.* (32) that MDMA blocks [^{3}H]paroxetine binding to serotonin transporters in brain. The concentration of MDMA required for displacing imipramine is higher than that observed for inhibiting transport or stimulating efflux. This phenomenon results from the kinetic characteristics of this transport system (31, 33, 34) in which a transported substrate or inhibitor has a lower K_m or K_i relative to its true dissociation constant measured in equilibrium binding studies.

The stereospecificity of the actions of MDMA at the serotonin transporter mirrors the higher potency of (+)-MDMA in behavioral studies (35). This contrasts with the higher potency of the R (−)-isomer for hallucinogenic amphetamines (36). Our results are, therefore, consistent with the possibility that behavioral effects of MDMA are mediated through its actions at the serotonin transporter.

Efflux from vesicular membranes is more complex, since MDMA dissipates the ΔpH (interior acid) in addition to its direct effect on the vesicular amine transporter. The ΔpH is generated by the vacuolar ATPase, which pumps H^+ ions into the vesicle interior (24). Serotonin accumulation is driven and maintained by this pH difference, and dissipation of ΔpH decreases serotonin accumulation and increases efflux. However, MDMA inhibits serotonin influx at concentrations where there is little or no dissipation of ΔpH. Furthermore, MDMA-induced efflux is stereospecific and is apparent at MDMA concentrations that do not significantly dissipate ΔpH. Thus, it is reasonable to conclude that direct interactions with the amine transporter, such as serotonin–MDMA exchange, are responsible for at least part of the MDMA-induced serotonin efflux from secretory vesicles. When acidified by ATP-dependent H^+ pumping, chromaffin granule membrane vesicles lose internal serotonin very slowly, since internal H^+ ions apparently compete with internal serotonin (37). This phenomenon is likely to account for the fact that MDMA induces significant efflux only at concentrations higher than those that inhibit [^{3}H]serotonin influx.

The ability of MDMA to dissipate ΔpH demonstrates that it is permeant, at least in its uncharged form. This ability of

MDMA to readily diffuse across membranes and its ability to serve as a transport substrate suggest a mechanism for MDMA-induced serotonin release by nerve terminals. Extracellular MDMA is expected to enter cells either passively or by way of the serotonin transporter and then enter synaptic vesicles in a process that consumes intravesicular H^+ ions. MDMA may cause serotonin efflux from the vesicle to the cytoplasm by increasing internal pH or by preferentially inhibiting influx relative to efflux, as was shown for tyramine (38). Before cytoplasmic serotonin can be oxidized by monoamine oxidase, exchange with extracellular MDMA catalyzed by the plasma membrane serotonin transporter leads to serotonin efflux from the cell. This process would explain the relative lack of 5-hydroxyindoleacetic acid in MDMA-treated animals compared to those treated with reserpine (39). Reserpine depletes vesicular monoamines but does not influence the plasma membrane transporter (14).

Other amphetamine derivatives that cause serotonin release, such as 3,4-methylenedioxyamphetamine, *p*-chloroamphetamine, and fenfluramine, may also be substrates for serotonin transport systems. The neurotoxic potential of these compounds may be related to their ability to exchange with serotonin or to dissipate transmembrane ion gradients and thereby tax energy metabolism in the nerve terminal. Moreover, drugs that release catecholamines, such as amphetamine and methamphetamine, may also act by depleting vesicular stores and exchanging through the plasma membrane catecholamine transport systems. Studies with synaptosomal preparations provide some support for this hypothesis (40, 41). Indeed, the specificity of plasma membrane biogenic amine transporters may play an important role in determining the sensitivity of a given aminergic neuron to amphetamines.

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