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Release of Dopamine via the Human Transporter

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SUMMARY

A human dopamine transporter cDNA was cloned and transfected into COS-7 cells, a cell line that lacks vesicular storage and release mechanisms. Cells expressing the dopamine transporter acquired the capacity to take up and release dopamine via the transporter. Ionic conditions that stimulate inside-out transport *in vivo*, such as depolarizing concentrations of K^+ or low concentrations of extracellular Na^+, were found to stimulate Ca^{2+}-independent release of [^{3}H]dopamine from transfected COS-7 cells. Dopamine uptake inhibitors had one of three effects on transporter-mediated efflux. Some drugs, in addition to inhibiting uptake, inhibited spontaneous release of dopamine. Drugs in this class included mazindol, GBR-12935, bupropion, nomifensine, and benztropine. All of the drugs with the potential for abuse by humans either enhanced release (methamphetamine, amphetamine, and ethanol) or had no effect on release (phencyclidine, cocaine, and WIN 35,428). The ability to define classes of uptake blockers based on their effects on human transporter-mediated dopamine efflux may lead to the identification of structural features of the transporter that differentiate abused from nonabused drugs.

The reinforcing properties of cocaine and many other abused drugs result from their interactions with the DAT and the subsequent increase in DA receptor stimulation (1–3). Drugs that bind to the DAT can modulate synaptic concentrations of DA in several ways. For example, passive blockade of the transporter by cocaine inhibits uptake into the presynaptic nerve terminal, resulting in increased synaptic DA concentrations (4). METH and amphetamine are substrates for DA, norepinephrine, and serotonin transporters (5) that increase the release of monoamines from neuronal preparations (6, 7) or platelet membrane vesicles (8) via exchange through the transporters, as well as competing for the binding site for monoamines on the transporters. Thus, the increase in the synaptic availability of DA has been suggested to underlie the abuse potential of cocaine, amphetamine, and other addictive substances.

However, several therapeutic drugs with a low incidence of abuse in humans also inhibit DA uptake. Mazindol, an appetite suppressant (9), and nomifensine, a novel bicyclic antidepressant (10), block both DA and norepinephrine transporters with high affinity (11). Two clinically effective drugs that exhibit high affinity and selective blockade of DA uptake are bupropion, a monocyclic antidepressant (12), and benztropine, an antimuscarinic compound used to block neuroleptic agent-induced extrapyramidal side effects. Thus, blockade of DA uptake is insufficient to predict abuse potential in humans.

The use of synaptosomes, brain slices, or *in vivo* microdialysis to investigate modulation of neurotransmitter release and uptake is complicated by multiple interactions between drugs and several cellular components, including transporters, receptors, and vesicular stores of amines. Tools for selective characterization of monoamine transporters have recently become available due to the cloning of cDNAs for the human transporters of DA, norepinephrine, and serotonin (13–16). We now report that the cloned hDAT mediates calcium-independent release of DA and that DA uptake inhibitors can be classified into three groups, based on their effects on release.

Materials and Methods

Cloning and expression of a hDAT cDNA. A 0.5-kilobase fragment of a rat DAT cDNA from base pairs 205 to 672 of the published sequence (17, 18) was amplified from rat brain cDNA by the polymerase chain reaction. The amplified fragment was radiolabeled by random hexamer-primed DNA synthesis and was used to screen a human substantia nigra cDNA library (Clontech). Five positive recombinants were selected and plaque-purified. The largest insert (3.5 kilobases) was subcloned into the eukaryotic expression vector pcDNA-1. The orientation of the insert was confirmed by restriction analysis with *Pst*I and *Bam*HI, and the insert was sequenced. COS-7 cells were transfected with pcDNA1-hDAT DNA using the calcium phosphate precipitation method of Chen and Okayama (19). On the day after

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ABBREVIATIONS: DAT, dopamine transporter; DA, dopamine; hDAT, human dopamine transporter; METH, methamphetamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

addition of the DNA mixture to the medium the cells were washed, detached from the tissue culture plate using a nonenzymatic cell dissociation solution (Sigma), and split into 24-well plates. COS-7 cells expressing the hDAT (COS-7-hDAT cells) were used for release assays on day 2 after transfection.

DA release. [^{3}H]DA (20 nM) was added to each well in Krebs-HEPES buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 μM pargyline, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid). Loading of the cells was conducted for 20 min, at which time the intracellular [^{3}H]DA concentration had reached steady state. Nonspecific uptake was defined as uptake in the absence of sodium or in the presence of 5 μM mazindol. The loading buffer was removed and cells were quickly washed with ice-cold release buffer (2 × 0.3 ml), which was identical to the loading buffer except that release buffer lacked Ca^{2+}. Ice-cold release buffer (0.5 ml) and drugs were added, and release was initiated by placing the tissue culture cluster in a 37° water bath. Release was terminated by aspiration of the buffer. Trichloroacetic acid (3%) was added and radioactivity remaining in the cells was determined by liquid scintillation counting. All experiments were conducted in triplicate. Data are expressed either as a percentage of the amount of [^{3}H]DA in the cells at time 0 (Fig. 1) or as a percentage of the amount of [^{3}H]DA in the cells after 3 min of release in the absence of drug (Figs. 3–5).

Experiments in which DA was measured by high performance liquid chromatography with electrochemical detection were carried out similarly, except that cells were loaded with unlabeled DA. The release buffer was removed from the cells and acidified by addition of 0.5 volume of 0.1 N HCl. DA remaining in the cells was extracted into 3% trichloroacetic acid. DA was extracted into alumina essentially as described by Refshauge *et al.* (20), using dihydroxybenzylamine as an internal standard, and was quantified exactly as described (21).

Results and Discussion

COS-7-hDAT cells that were preloaded with [^{3}H]DA released radioactivity spontaneously, as assessed by the reduction in [^{3}H]DA remaining in the cells (Fig. 1). The addition of METH accelerated the release of [^{3}H]DA. At 3 min, the control cells retained 77.9 ± 8.5% of preloaded [^{3}H]DA, whereas METH-treated cells retained 48.1 ± 3.5% of preloaded [^{3}H]DA. Other conditions that decreased the amount of preloaded [^{3}H]DA retained in the cells included the addition of 100 mM K^+ (48.3 ± 2.3% retained) or the replacement of extracellular Na^+ with choline in the release buffer (38.0 ± 2.8% retained). The finding that release was accelerated in the presence of a depolarizing concentration of K^+ or as a result of reversing the Na^+ gradient indicates that this system responds to the same electrochemical gradients that induce transporter reversal in brain preparations (6, 22–24). Therefore, spontaneous and drug-induced release of [^{3}H]DA from preloaded COS-7-hDAT cells occurs in a calcium-independent manner, reflecting reversal of transporter action (25, 26). Because the magnitude of the difference between spontaneous and stimulated release was greatest at 1–3 min after the initiation of release, additional experiments with [^{3}H]DA were carried out for 3 min and were analyzed by expressing the cellular radioactivity measured after incubation in the presence of drug as a percentage of cellular radioactivity remaining after incubation with no drug.

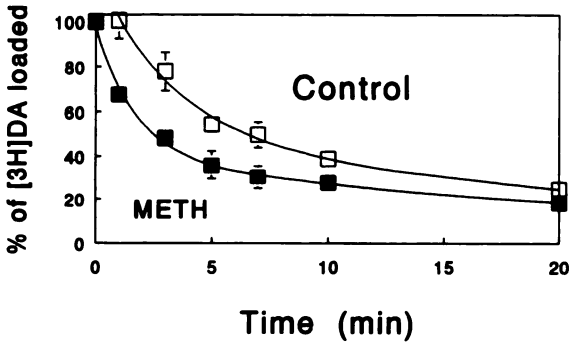

Fig. 1. Time course of increased release of [^{3}H]DA from COS-7-hDAT cells by METH (1 mM). The data are normalized to the amount of [^{3}H]DA in the cells at time 0. All release experiments were conducted in the absence of calcium. Data are the mean ± standard error from three experiments.

To confirm that decreased intracellular radioactivity resulted from increased release of DA, the DA content of the cells and buffer was measured using COS-7-hDAT cells loaded with unlabeled DA. Transformation of the data in Fig. 2 to the percentage of cellular DA released [buffer DA/(buffer DA + cellular DA)] indicated that under basal conditions 34% of total DA was in the buffer by 3 min, whereas in the presence of METH 69% of total DA was in the buffer. The METH-induced increase in the DA content of the buffer was associated with a corresponding decrease in the cellular content of DA. Consequently, measuring the decrease in [^{3}H]DA content of the cells is a valid determination of [^{3}H]DA release.

Inhibitors of DA uptake had three different effects on the release of [^{3}H]DA (Fig. 3; Table 1). First, a number of compounds that are substrates for the transporter (METH, amphetamine, DA, tyramine, and serotonin) caused a concentration-dependent enhancement of [^{3}H]DA release. METH was most potent, with an EC_{50} of 0.2 μM. METH and the monoamines DA, tyramine, and serotonin were the most efficacious compounds tested, with maximal stimulated release being approximately 50% greater than spontaneous release. Ethanol also enhanced release at a physiologically relevant concentration (10 mM; 0.06%, v/v), suggesting that it interacts with the hDAT by a mechanism similar to that of substrates.

Typifying the second effect, the selective DAT ligand GBR-12935 (27, 28) inhibited [^{3}H]DA release at concentrations up to 30 nM. In some experiments, 30 nM GBR-12935 inhibited all spontaneous release. Higher concentrations of GBR-12935 had decreasing efficacy; because 95% of GBR-12935 hDAT binding sites are occupied at 30 nM, the decrease in efficacy

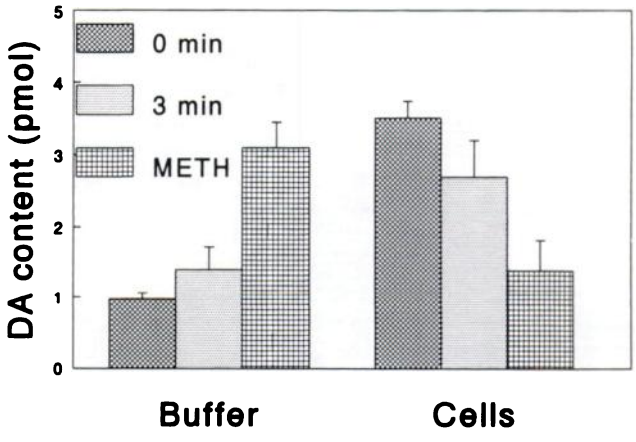

Fig. 2. Effect of METH on DA content in COS-7-hDAT cells and buffer. DA was measured by high performance liquid chromatography with electrochemical detection, as described in Materials and Methods. Data are expressed as mean ± standard error of pmol of DA released (buffer) or retained (cells) in the well. Similar results were obtained in a second independent experiment.

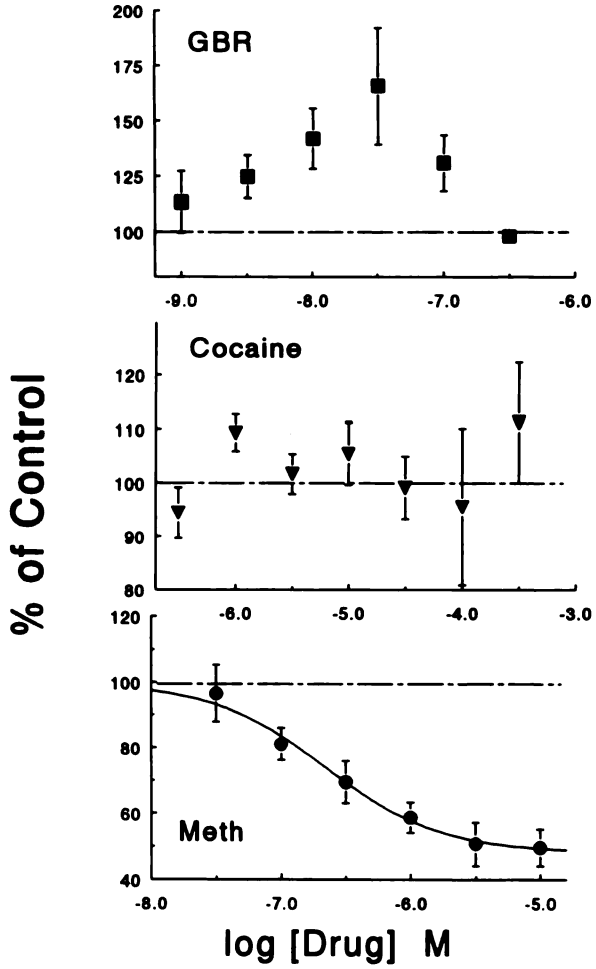

Fig. 3. Drug modulation of spontaneous [^{3}H]DA release from COS-7-hDAT cells. Concentration-response curves were conducted for GBR-12935 (*GBR*) (*top*), cocaine (*middle*), and METH (*bottom*). Release was carried out for 3 min and terminated by aspiration of release buffer. The [^{3}H]DA remaining in the cells in the presence of a given drug is expressed as a percentage of [^{3}H]DA remaining in the cells in the absence of drug (spontaneous release). The average spontaneous release of [^{3}H]DA in each set of experiments was 46 ± 2%, 45 ± 4%, and 36 ± 5%, respectively. Data were analyzed by the nonlinear curve-fitting program GraphPAD.

TABLE 1

Effects of drugs on spontaneous [^{3}H]DA release

EC_{50} (enhancers) and IC_{50} (inhibitors) values were calculated by nonlinear regression. The maximal effect was calculated as the radioactivity remaining in the cells in the presence of drug, expressed as a percentage of the radioactivity in the absence of drug. Results from at least three independent experiments for each drug were averaged and analyzed as a single curve to yield the values listed. For drugs that had little effect, the range of tested concentrations is given.

Drug	Potency	Maximal effect
	μM	%
Release enhancers		
(+)-METH	0.2	52
Amphetamine	1.7	42
DA	4.9	49
Tyramine	6.4	49
Serotonin	45	53
Ethanol	10,000	19
Release inhibitors		
GBR-12935	0.005	65
Mazindol	0.003	26
Benztropine	0.005	33
Nomifensine	0.03	52
Bupropion	0.19	38
Drugs with little or no effect		
Cocaine	0.03–300	
WIN 35,428	0.003–1	
threo-(±)-Methylphenidate	0.03–10	
Desipramine	0.3–100	
Phencyclidine	0.3–100	

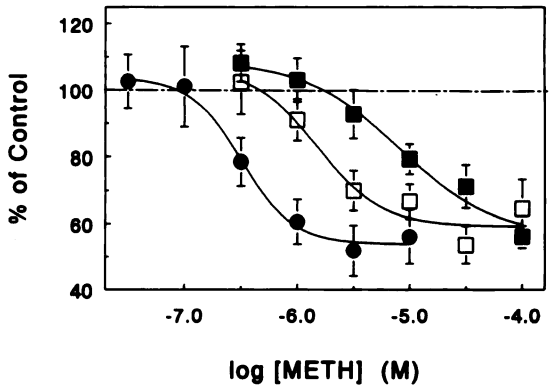

Fig. 4. Inhibition by cocaine and GBR-12935 of METH-induced release of [^{3}H]DA. Release was measured as in Fig. 3. The average spontaneous release of [^{3}H]DA in these experiments was 48 ± 3%. ●, METH; □, METH plus 30 nM GBR-12935; ■, METH plus 10 μM cocaine. The data shown are the mean ± standard error of three to five experiments.

may not be related to the binding of GBR-12935 to the hDAT. Other drugs that inhibited release were compounds that are or have been used clinically, including mazindol, benztropine, nomifensine, and bupropion (29). The similarity between potencies for inhibition of release (Table 1) and inhibition of DA uptake (15) further demonstrates that spontaneous release is via the hDAT.

A third group of [^{3}H]DA uptake blockers, including cocaine, the cocaine analog WIN 35,428, methylphenidate, and phencyclidine, were without significant effect on [^{3}H]DA release at concentrations that bracketed their IC_{50} values for inhibition of uptake.[1] Except for the selective norepinephrine uptake blocker desmethylimipramine, drugs in this category have abuse potential (1, 2).

The effect of cocaine or GBR-12935 on METH-induced release of [^{3}H]DA is shown in Fig. 4. The EC_{50} value for METH in this set of experiments was 315 ± 4.4 nM, which increased to 1.46 ± 0.11 μM and 8.11 ± 2.25 μM in the presence of 30 nM GBR-12935 and 10 μM cocaine, respectively. The decrease in potency with no change in the maximal effect appears consistent with competitive inhibition. However, both METH and cocaine prevented the inhibition of spontaneous release of [^{3}H]DA by GBR-12935, a blockade that was insurmountable by any concentration of GBR-12935 (Fig. 5). The inability of GBR-12935 to overcome the blockade by cocaine and METH supports the contention that the interaction between the binding sites for METH or cocaine and GBR-12935 is not competitive (30, 31).

Transporter-mediated release of monoamines that is stimulated by amphetamine or related drugs has been described for a number of preparations, including tissue slices (7, 22), brain homogenates or synaptosomes (6, 24, 32), dissociated neurons

[1] For the compounds that had no effect on release, the IC_{50} values for inhibition of [^{3}H]DA uptake were as follows: WIN 35,428, 14.7 nM; methylphenidate, 143 nM; cocaine, 200 nM; phencyclidine, 734 nM; and desmethylimipramine, 13,500 nM.

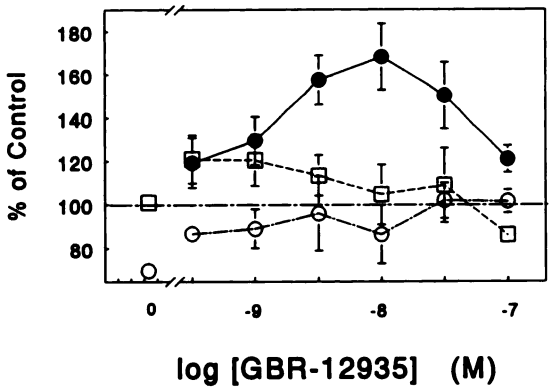

Fig. 5. Modulation by cocaine and METH of release inhibited by GBR-12935. Release was measured as in Fig. 3. The average spontaneous release of [^{3}H]DA in these experiments was 41 ± 5%. ●, GBR-12935; □, GBR-12935 plus 10 μM cocaine; ○, GBR-12935 plus 300 nM METH. The data shown are the mean ± standard error of four to six experiments.

(33, 34), platelet membrane vesicles (8, 35), and cells stably expressing a recombinant DAT (8). Our finding that DA uptake blockers inhibit METH-induced release of [^{3}H]DA is consistent with prior studies (6, 22, 24, 32). Amphetamine-like compounds release monoamines via effects on both membrane transporters and vesicular stores of amines (8, 33–35). COS-7 cells presumably lack a vesicular storage system for monoamines, as suggested by their sensitivity to 1-methyl-4-phenylpyridinium toxicity (36) after transfection with a DAT cDNA (37). The lack of sequestration of cytosolic DA into vesicles in COS-7 cells, and the resulting higher inside-out concentration gradient, could explain the substantial spontaneous [^{3}H]DA efflux observed. It is this spontaneous efflux that makes it possible to differentiate uptake blockers that do not stimulate efflux into those that inhibit release and those with no effect on release.

One interpretation of these results is that compounds that inhibit uptake and spontaneous release of [^{3}H]DA are "pure" antagonists. If the DA binding site of the transporter can face the inner or outer surface of the membrane (7, 8), with the direction of DA transport being dependent on which way the binding site is facing, then a pure antagonist such as mazindol may have higher affinity for the outward-facing conformation. The binding of mazindol would then shift the equilibrium towards that conformation, preventing binding of intracellular DA to the DAT and outward transport. At the same time, the antagonist would competitively inhibit binding of DA to the outward-facing binding site. In contrast, a substrate of the transporter such as METH inhibits DA uptake by competing for outward-facing DA binding sites and also stimulates release by increasing the rate of return of the transporter to an inward-facing position, thus allowing binding and outward transport of DA (6–8). Drugs in the third class, including cocaine and methylphenidate, could have similar affinities for the hDAT in outward- or inward-facing conformations, so that they do not alter the equilibrium between the conformations. These drugs may inhibit binding of extracellular DA, and thus inhibit inward transport (uptake), without preventing the binding of intracellular DA, thus allowing spontaneous outward transport (release). Because cocaine does not alter spontaneous DA release (Fig. 3), our data further suggest that cocaine inhibition of METH-induced release (Fig. 4) is via blockade of METH uptake or binding to the transporter, rather than inhibition of the outward flow of DA.

Selective modulation of DA efflux may be relevant to the potential for a drug to be abused. For example, inhibition of DA release via the DAT is a potential mechanism for attenuation of cocaine-induced increases in extracellular DA *in vivo*, and antagonists that inhibit release may be clinically useful for antagonizing the effects of abused substances (29). Mazindol, which inhibits both uptake and release of [^{3}H]DA, decreases craving associated with cocaine abuse (38); although it causes similar behaviors as does cocaine in animals, it is dysphoric in normal human volunteers (9). Nearly all DA uptake blockers are self-administered by laboratory animals (1, 2, 9, 39), so it is possible that differentiating among drugs may be a property unique to the hDAT.

The ability to differentiate among three different classes of drugs is a novel result of these experiments. All of the drugs tested appear qualititatively similar as inhibitors of DA uptake, although they differ in potency. Similarly, differences assessed by characterization of radioligand binding are subtle and controversial (compare Refs. 30 and 40). The finding of qualitative differences in the effects of drugs on spontaneous hDAT-mediated DA efflux makes it possible to identify the structural features of the transporter that are involved in selective interactions with abused or nonabused drugs.

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