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Amphetamine-Type Central Nervous System Stimulants Release Norepinephrine More Potently Than They Release Dopamine and Serotonin

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ABSTRACT A large body of evidence supports the hypothesis that mesolimbic dopamine (DA) mediates, in animal models, the reinforcing effects of central nervous system stimulants such as cocaine and amphetamine. The role DA plays in mediating amphetamine-type subjective effects of stimulants in humans remains to be established. Both amphetamine and cocaine increase norepinephrine (NE) via stimulation of release and inhibition of reuptake, respectively. If increases in NE mediate amphetamine-type subjective effects of stimulants in humans, then one would predict that stimulant medications that produce amphetamine-type subjective effects in humans should share the ability to increase NE. To test this hypothesis, we determined, using in vitro methods, the neurochemical mechanism of action of amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), (+)-methamphetamine, ephedrine, phentermine, and aminorex. As expected, their rank order of potency for DA release was similar to their rank order of potency in published self-administration studies. Interestingly, the results demonstrated that the most potent effect of these stimulants is to release NE. Importantly, the oral dose of these stimulants, which produce amphetamine-type subjective effects in humans, correlated with the their potency in releasing NE, not DA, and did not decrease plasma prolactin, an effect mediated by DA release. These results suggest that NE may contribute to the amphetamine-type subjective effects of stimulants in humans. **Synapse 39:32–41, 2001.** Published 2001 Wiley-Liss, Inc.†

INTRODUCTION

A large body of evidence supports the hypothesis that mesolimbic dopamine (DA) mediates the reinforcing effects of central nervous system stimulants such as cocaine and amphetamine (Wise, 1996; Kuhar et al., 1991; Johanson and Fischman, 1989). Although it is tempting to assume that DA also mediates the amphetamine-type subjective effects of these medications in humans, published data do not support this hypothesis. These data, as reviewed in detail elsewhere (Rothman and Glowa, 1995; Rothman, 1994; Brauer et al., 1997; Villemagne et al., 1999), include the observations that DA receptor antagonists do not block the subjective effects of cocaine (Ohuoha et al., 1997; Malison et al., 1997; Price et al., 1997) or amphetamine (Brauer and de Wit, 1997, 1996). These and other data reviewed in a recent publication (Villemagne et al., 1999) suggest that increases in brain DA may be necessary, but not sufficient, to produce the amphetamine-type subjective effects of these agents in humans. In particular, both amphetamine (Segal and Kuczenski, 1997) and cocaine (Reith et al., 1997) increase norepinephrine (NE) via stimulation of release and inhibition of reuptake, respectively. If increases in NE contribute to the amphetamine-type subjective effects of stimulants, then one would predict that stimulant medications that produce amphetamine-type subjective effects in humans should share the ability to increase NE. To test this hypothesis, we first determined the neurochemical mechanism of action of well-studied stimulants such as

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amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and (+)-methamphetamine ((+)-METH) as well as stimulants which have received scant study with modern neurochemical methods: ephedrine, phentermine, and aminorex. Using published data, we then correlated the potency of the agents for releasing NE with the oral dose which produces amphetamine-type subjective effects in humans.

Drugs that interact with the integral membrane proteins that function as transporters for the biogenic amines DA, NE, and serotonin (5-HT) can be divided into two groups. Uptake inhibitors bind to the transporter, yet are not transported. These agents elevate synaptic neurotransmitters by interfering with their removal from the synaptic extracellular space. Substrates, in contrast, are transported by these proteins into the nerve terminal, where they promote the release of neurotransmitter by a two-pronged mechanism. Substrates increase cytoplasmic neurotransmitter by interfering with the accumulation of neurotransmitter in storage vesicles and they promote a process of carrier-mediated exchange (Rudnick and Clark, 1993). As noted above, many stimulants developed in the 1950s and 1960s have not been studied with modern neurochemical methods and could act either as uptake inhibitors or substrates of the biogenic amine transporters. We therefore characterized the actions of these agents using an in vitro method that discriminates between transporter uptake inhibitors and substrates.

MATERIALS AND METHODS

[^{3}H]DA, [^{3}H]5-HT and [^{3}H]NE reuptake assays

The effect of test agents on [^{3}H]DA and [^{3}H]5-HT uptake was evaluated using published methods (Rothman et al., 1993). Briefly, synaptosomes were prepared from rat caudate for [^{3}H]DA reuptake or from whole rat brain minus caudate and cerebellum for [^{3}H]5-HT reuptake. Fresh tissue was homogenized in ice-cold 10% sucrose using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1,000*g* for 10 min at 4°C and supernatants were retained on ice. Polystyrene test tubes (12 × 75 mm) were prefilled with 50 μl of Krebs-phosphate buffer (final pH = 7.4) consisting of 0.5 mM Na_2SO_4, 0.5 mM KH_2PO_4, 126 mM NaCl, 2.4 mM KCl, 0.83 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11.1 mM glucose at pH 7.4, with 1 mg/ml ascorbic acid, 1 mg/ml bovine serum albumin (BSA), and 50 μM pargyline added (uptake buffer), 750 μl of [^{3}H]DA (5 nM) or [^{3}H]5-HT (2 nM) diluted in uptake buffer without BSA, and 100 μl of test agent in uptake buffer. Nonspecific uptake was defined using 10 μM tyramine ([^{3}H]DA) or 100 μM tyramine ([^{3}H]5-HT).

[^{3}H]NE uptake proceeded with minor modifications of the above procedure: the tissue source was whole rat brain minus caudate and cerebellum, 5 nM RTI-229 was used to block uptake of [^{3}H]NE into DAergic nerves, the incubations proceeded for 10 min at 25°C, the concentration of [^{3}H]NE was 5 nM, and nonspecific uptake was defined using 10 μM indatraline or 10 μM tyramine.

The uptake assay was initiated by adding 100 μl of the synaptosomal preparation to the tubes. Inhibition curves were generated by incubating [^{3}H]ligand with test agent (1 nM to 100 μM final tube concentration) diluted in uptake buffer. The [^{3}H]5-HT reuptake experiments were conducted in the presence of 100 nM nomifensine and 100 nM GBR12935 to prevent uptake into noradrenerigic or DA nerve terminals. Incubations of 15 or 30 min were carried out at 25°C for [^{3}H]DA and [^{3}H]5-HT, respectively. The incubations were terminated by adding 4 ml of wash buffer containing 10 mM Tris HCl (pH 7.4) in 0.9% NaCl at 25°C, followed by rapid filtration over Whatman GF/B filters and two additional wash cycles. The tritium retained on the filters was counted in a beta counter (Taurus, Titertek, Huntsville, AL) at 40% efficiency after an overnight extraction into ICN Cytoscint cocktail (ICN Biomedicals, Costa Mesa, CA).

[^{3}H]DA, [^{3}H]NE and [^{3}H]5-HT release assays

Rat caudate (for [^{3}H]DA release) or whole brain minus cerebellum and caudate (for [^{3}H]NE and ^{3}H]5HT release) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. Nomifensine (100 nM) and GBR12935 (100 nM) were also added to the sucrose solution for [^{3}H]5HT release experiments to block any potential [^{3}H]5HT reuptake into NE and DA nerve terminals. After 12 strokes with a Potter-Elvehjem homogenizer, homogenates were centrifuged at 1,000*g* for 10 min at 0–4°C and the supernatants were retained on ice (synaptosomal preparation). Each rat brain (approximately 1,200 mg) produced enough tissue preparation for 250 test tubes for the [^{3}H]DA and [^{3}H]5-HT release assays and for 125 test tubes for the [^{3}H]NE release assay.

Synaptosomal preparations were incubated to steady-state with 5 nM [^{3}H]DA (30 min), 7 nM [^{3}H]NE (60 min), or 5 nM [^{3}H]5HT (60 min) in uptake buffer (without BSA) plus 1 μM reserpine in a polypropylene beaker with stirring at 25°C. Nomifensine (100 nM) and GBR12935 (100 nM) were added to the buffer for [^{3}H]5HT release experiments. RTI-229 (5 nM) was added to the buffer for [^{3}H]NE release experiments to prevent [^{3}H]NE reuptake into dopamine nerve terminals. After incubation to steady-state, 850 μl of synaptosomes preloaded with [^{3}H]neurotransmitter were added to 12 × 75 mm polystyrene test tubes which contained 150 μl test drug in uptake buffer. After 5 min ([^{3}H]DA and [^{3}H]5-HT) or 30 min ([^{3}H]NE) the release reaction was terminated by dilution with 4 ml wash buffer (10 mM Tris-HCl pH 7.4 containing 0.9% NaCl at 25°C) followed by rapid vacuum filtration over Whatman GF/B filters using a Brandel Harvester. The filters were rinsed twice with 4 ml wash buffer using the Brandel Harvester and the retained tritium was

TABLE I. Representative signal-to-noise ratios observed in the release assays

Assay	Total retained tritium (CPM)	Nonspecific binding (CPM)	Specific (CPM)	Total/ nonspecific
[^{3}H]NE	3,000	1,500	1,500	2.0
[^{3}H]DA	3,700	1,900	1,800	1.9
[^{3}H]5-HT	13,000	6,500	6,500	2.0

Representative counts per minute (cpm) obtained when the release assays are conducted as described in Methods.

counted by a Taurus liquid scintillation counter at 40% efficiency after an overnight extraction in 3 ml Cytoscint (ICN). Typical signal-to-noise ratios are reported in Table I.

Data analysis and statistics

As previously described (Rothman et al., 1993), IC_{50} values were determined using the nonlinear least-squares curve-fitting program MLAB-PC (Civilized Software, Bethesda, MD). In uptake experiments, K_i values were calculated according to the formula (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + L/Km)$ where L is the concentration of the radiolabeled drug ([^{3}H]DA, [^{3}H]NE or [^{3}H]5-HT). In the [^{3}H]NE uptake assays, surfaces were fit to one- and two-component models (Akunne et al., 1994). The F-test was used to determine the better-fitting model. In release experiments, the apparent K_i of antagonists was calculated according to the following formula: K_i = [Antagonist]/(IC_{50_2}/IC_{50_1} − 1) where IC_{50_1} is the IC_{50} in the absence of antagonist and IC_{50_2} is the IC_{50} in the presence of antagonist.

Drugs and reagents

(+)-Fenfluramine HCl, (±)-fenfluramine, chlorphentermine HCl (FW = 220.2), (+)-amphetamine sulfate, phentermine HCl, (±)-MDMA, (−)-METH, and (+)-METH were obtained from the Addiction Research Center Pharmacy (NIDA, NIH, Baltimore, MD). Aminorex, (−)-norepinephrine, dopamine, desipramine, fluoxetine, (−)-ephedrine, tyramine, and indatraline were purchased from Research Biochemicals (Natick, MA). RTI-55 and RTI-229 were provided by Dr. F. Ivy Carroll. [^{3}H]DA (SA = 27.5 Ci/mmol), [^{3}H]NE (SA = 55 Ci/mmol) and [^{3}H]5-HT (SA = 27.5 Ci/mmol) were purchased from Dupont New England Nuclear (Boston, MA). The sources of other reagents are published (Rothman et al., 1993, 1994).

RESULTS

[^{3}H]NE uptake experiments

Initial experiments indicated that [^{3}H]NE uptake was composed of two components. To characterize these components, uptake "surfaces" were generated according to the experimental design outlined in Table II. Two concentrations of [^{3}H]NE (5 nM and 105 nM) were each inhibited by NE, the NE-selective uptake inhibitor desipramine, and the DA-selective uptake inhibitor RTI-229 in the absence and presence of the indicated concentrations of "blocking" agents.

TABLE II. Experimental design used to characterize [^{3}H]NE uptake

Surface/[NE] nM	Primary inhibitor	Blocker
1	NE	None
5 nM [3H]NE	(10–10,000 nM)	
2	NE	None
5 nM [3H]NE + 100 nM NE	(10–10,000 nM)	
3	NE	25 nM
5 nM [3H]NE	(10–10,000 nM)	Desipramine
4	NE	25 nM
5 nM [3H]NE + 100 nM NE	(10–10,000 nM)	Desipramine
5	NE	20 nM RTI229
5 nM [3H]NE	(10–10,000 nM)	
6	NE	20 nM RTI229
5 nM [3H]NE + 100 nM NE	(10–10,000 nM)	
7	Desipramine	None
5 nM [3H]NE	(0.5–50,000 nM)	
8	Desipramine	None
5 nM [3H]NE + 100 nM NE	(0.5–50,000 nM)	
9	Desipramine	20 nM RTI229
5 nM [3H]NE	(0.5–50,000 nM)	
10	Desipramine	20 nM RTI229
5 nM [3H]NE + 100 nM NE	(0.5–50,000 nM)	
11	RTI229	None
5 nM [3H]NE	(0.1–1,000 nM)	
12	RTI229	None
5 nM [3H]NE + 100 nM NE	(0.1–1,000 nM)	
13	RTI229	25 nM
5 nM [3H]NE	(0.1–1,000 nM)	desipramine
14	RTI229	25 nM
5 nM [3H]NE + 100 nM NE	(0.1–1,000 nM)	desipramine

Uptake "surfaces" were generated according to the experimental design described above. Two concentrations of [^{3}H]NE (5 nM and 105 nM) were each inhibited by eight concentrations of NE, the NE-selective uptake inhibitor desipramine, and the DA-selective uptake inhibitor RTI-229 in the absence and presence of the indicated concentrations of "blocking" agents.

TABLE III. Best-fit parameter estimates for [^{3}H]NE uptake inhibition

Parameter	NE transporter	DA transporter
Vmax (fmol/mg protein)	2,600 ± 50	10,200 ± 350
NE (Km, nM)	63.9 ± 1.6	706 ± 35
Desipramine (K_i, nM)	1.03 ± 0.05	7,220 ± 690
RTI-229 (K_i, nM)	19.5 ± 0.6	0.35 ± 0.02

The data of the three experiments described in Table II were pooled to yield 504 points. The entire set of data was fit to a one-component model (sum-of-squares = 8,220) and a two-component model (sum-of-squares = 536). The two-component model fit significantly better than the one-component model ($P < 0.001$). Each value is ±SD.

As reported in Table III, [^{3}H]NE uptake inhibition fit a two-component model. NE had the higher affinity for the lower capacity component (Km = 63.9 nM) and lower affinity for the higher capacity component (Km = 706 nM). The high affinity and selectivity of desipramine for the lower capacity component identifies this as the NE transporter. The high affinity and selectivity of RTI-229 for the higher capacity component identifies this as the DA transporter.

Initial release experiments

Time-course experiments with [^{3}H]NE were conducted in the absence and presence of 1 μM reserpine to determine the time required to reach steady-state. As reported in Figure 1A, [^{3}H]NE failed to achieve

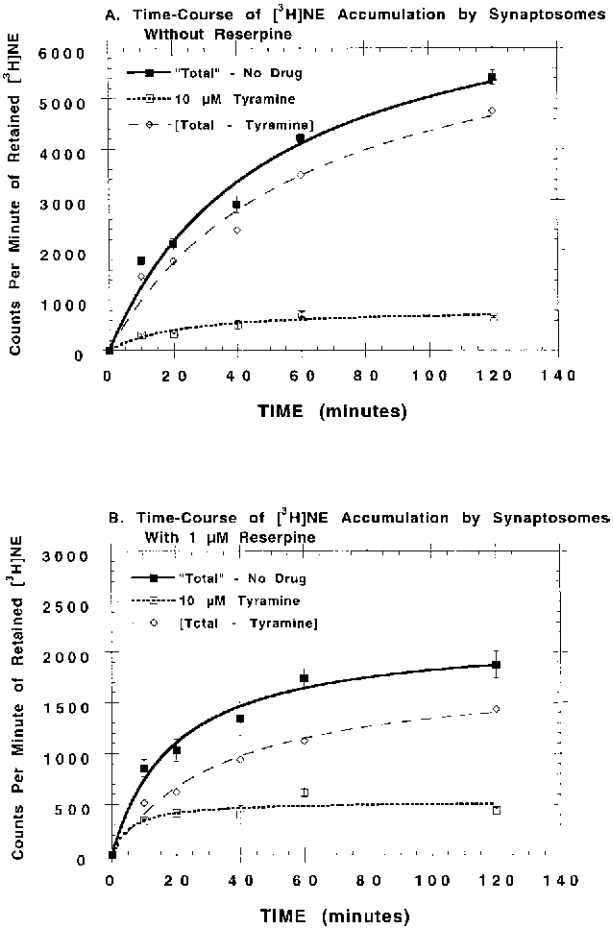


Fig. 1. Time-course of [^{3}H]NE accumulation by rat brain synaptosomes. **A:** In the absence of reserpine. **B:** In the presence of 1 µM reserpine. Each point is the mean ± SD ($n = 3$).

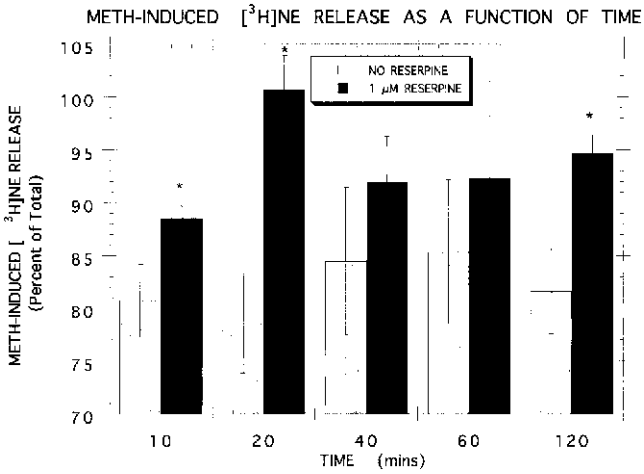


Fig. 2. Effect of (+)-METH on [^{3}H]NE release as a function of time in the absence and presence of 1 µM reserpine. Each point is the mean ± SD ($n = 3$). *$P < 0.05$ when compared to the no reserpine control (two-tailed t-test).

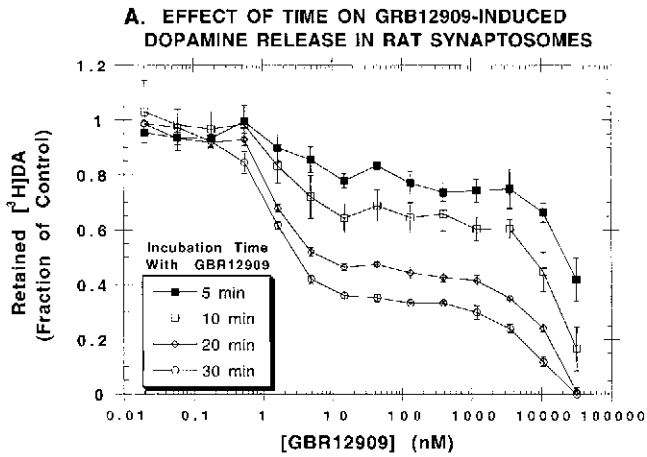
steady-state by 60 min in the absence of reserpine, but did so by 60 min in the presence of 1 µM reserpine (Fig. 1B). Reserpine reduced retained [^{3}H]NE by 65% at 60 min and increased the ability of (+)-METH to release [^{3}H]NE (Fig. 2). We therefore conducted reuptake time-course experiments with [^{3}H]5-HT and [^{3}H]DA in the presence of reserpine. These data (not shown) demonstrated that steady-state was achieved by 30 min with 5 nM [^{3}H]DA and by 60 min with 5 nM [^{3}H]5HT. Other experiments showed that accumulation of [^{3}H]neurotransmitter at steady-state was directly proportional to protein (data not shown). Typical protein concentrations used were: 0.75 mg/tube for [^{3}H]NE, 0.35 mg/tube for [^{3}H]5-HT, and 0.07 mg/tube for [^{3}H]DA.

Effect of incubation time on drug-induced [^{3}H]DA release

To determine the effect of incubation time on drug-induced [^{3}H]DA release, dose–response curves were generated with GBR12909, a DA uptake inhibitor, and (+)-METH, a DA releaser, with the incubations being terminated after 5, 10, 15, or 30 min. As reported in Figure 3A, longer incubation times with GBR12909 increased the potency of GBR12909 for stimulating [^{3}H]DA release. The release curve was biphasic with the level of the plateau increasing with shorter time incubations. With a 5-min incubation, even high concentrations of GBR12909 released no more than 20% of accumulated [^{3}H]DA. In contrast, as reported in Figure 3B, (+)-METH-induced [^{3}H]DA release was almost identical at all incubation times and complete release was achieved. Based on these results, [^{3}H]DA and [^{3}H]5-HT release assays were terminated after a 5-min incubation with test drugs. Similar experiments demonstrated that a 30-min incubation with test drugs was optimum for the [^{3}H]NE release assay (data not shown).

Pharmacological blockade of release

Uptake inhibitors block the releasing effect of substrates by preventing the entry of transporter substrates into the nerve terminal (Hurd and Ungerstedt, 1989a). To see whether this blockade also occurred with the in vitro release assays, substrate dose–response curves were generated in the absence and presence of low concentrations of selective uptake inhibitors. As reported in Table IVA, concentrations of the NE-selective uptake inhibitor desipramine (DMI) which did not alter [^{3}H]NE release, increased the IC_{50} values for (+)-METH-induced [^{3}H]NE release. Similarly, concentrations of the DA-selective uptake inhibitor GBR12909 which did not alter [^{3}H]DA release, increased the IC_{50} values for (+)-METH-induced [^{3}H]DA release (Table IVB). Finally, as shown in Table IVC, concentrations of the 5-HT-selective uptake inhibitor fluoxetine which did not alter [^{3}H]5-HT release increased the IC_{50} values for (+)-fenfluramine-induced [^{3}H]5-HT release. Based on the shift to the right in the



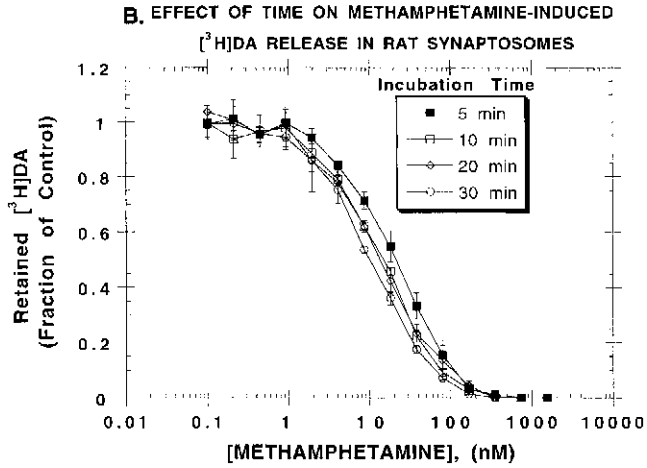


Fig. 3. Effect of time on GBR12909-induced (**A**) and (+)-METH-induced (**B**) [^{3}H]DA release. Each point is the mean ± SD (n = 3).

dose–response curves, it was possible to calculate an apparent functional K_i value for each uptake inhibitor. The apparent K_i values of DMI (about 3 nM), GBR12909 (about 0.3 nM) and fluoxetine (about 3 nM) were similar to their K_i values for inhibiting the uptake of [^{3}H]NE, [^{3}H]DA and [^{3}H]5-HT, respectively (Table V).

Pharmacological profile of CNS stimulants and related drugs

We determined the activity of a variety of transporter ligands, both uptake inhibitors and substrates, at [^{3}H]NE release and uptake, [^{3}H]DA release and uptake, and [^{3}H]5-HT release and uptake (Table V). DA-selective uptake inhibitors such as GBR12909, GBR12935, and RTI-229 were all inactive in the release assays. 5-HT-selective uptake inhibitors such as fluoxetine and citalopram were also inactive in the release assays. Similarly, the NE-selective uptake inhibitors

TABLE IV. Effect of uptake inhibitors on [^{3}H]neurotransmitter release

A. Effect of DMI on (+)-METH-induced [^{3}H]NE release

[DMI]	(+)-METH (IC_{50} ± SD)	N ± SD	DMI apparent Ki (nM)
0	10.6 ± 0.6	0.89 ± 0.04	
1 nM	15.7 ± 1.1	0.86 ± 0.05	2.1
10 nM	36.8 ± 5.8	0.70 ± 0.08	14.9
100 nM	249 ± 35	0.78 ± 0.09	4.4

B. Effect of GBR12909 on (+)-METH-induced [^{3}H]DA release

GBR12909 (nM)	(+)-METH (IC_{50} ± SD)	N ± SD	GBR12909 apparent Ki (nM)
0	18.1 ± 1.4	0.92 ± 0.06	
5	288 ± 30	1.08 ± 0.11	0.33nM
20	1,450 ± 128	0.91 ± 0.07	0.25nM

C. Effect of fluoxetine on (+)-fenfluramine-induced [^{3}H]5-HT release

[Fluoxetine]	(+)-Fenfluramine (IC_{50} ± SD)	N ± SD	Fluoxetine apparent Ki (nM)
0	45.9 ± 11.8	0.89 ± 0.10	
10 nM	135 ± 10	1.00 ± 0.07	5.1
100 nM	2,305 ± 194	0.75 ± 0.05	2.0

Dose–response curves were generated using 10 concentrations of substrate. The data from three experiments were pooled (30 points) and fit to the two parameter logistic equation for the best-fit estimates of the IC_{50} and slope factor (N). DMI had no effect on [^{3}H]NE release. GBR12909 at 5 nM and 20 nM inhibited retained [^{3}H]DA by 15%. Fluoxetine at 10 nM and 100 nM inhibited retained [^{3}H]5-HT by 3% and 7% respectively. Each value is the mean ± SD of three experiments. r^2 values were greater than 0.93.

desipramine and mazindol were inactive in the release assays. Nonselective uptake inhibitors such as cocaine and RTI-55 were also inactive in the release assays.

The endogenous substrates of the transporters were also tested. 5-HT potently inhibited [^{3}H]5-HT uptake (Ki = 16.7 nM) and was about 160-fold less potent at inhibiting [^{3}H]DA and [^{3}H]NE uptake. Consistent with these data, 5-HT potently released [^{3}H]5-HT (K_i = 44.4 nM) and was essentially inactive as a releaser of [^{3}H]NE and [^{3}H]DA. Both NE and DA weakly inhibited [^{3}H]5-HT uptake and were inactive as releasers of [^{3}H]5-HT. DA more potently released [^{3}H]NE (IC_{50} = 66.2 nM) than [^{3}H]DA (IC_{50} = 86.9 nM). In contrast, NE more potently released [^{3}H]NE (IC_{50} = 164 nM) than [^{3}H]DA (IC_{50} = 869 nM). Of note (see Discussion), DA was potently accumulated by noradrenergic nerves.

In contrast to uptake inhibitors, which were essentially inactive in the release assays, compounds known to be substrates were active in both uptake and release assays, although they were generally 5–10-fold more potent in the release assays. (+)-METH was most potent at NE release (IC_{50} = 12.3 nM), followed by DA release (IC_{50} = 24.5 nM), and 5-HT release (IC_{50} = 736 nM). (+)-Amphetamine was more potent than (+)-METH and had a similar profile: NE release (IC_{50} = 7.1 nM), DA release (IC_{50} = 24.8 nM), and 5-HT release (K_i = 1,765 nM). Ephedrine was a potent and selective releaser of NE: NE release (K_i = 72.4 nM), DA release (IC_{50} = 1,350 nM), and 5-HT release

TABLE V. Pharmacological profile of selected agents in the DA, NE and 5-HT release and uptake inhibition assays

Drug	NE release IC_{50} (nM ± SD)	NE uptake *Ki* (nM ± SD)	5-HT release IC_{50} (nM ± SD)	5-HT uptake *Ki* (nM ± SD)	DA release IC_{50} (nM ± SD)	DA uptake *Ki* (nM ± SD)
Aminorex	26.4 ± 2.8	54.5 ± 4.8	193 ± 23	1,244 ± 106	49.4 ± 7.5	216 ± 7
Chlorphentermine	>10,000	451 ± 66	30.9 ± 5.4	338 ± 6	2,650 ± 273	3,940 ± 110
Phentermine	39.4 ± 6.6	244 ± 15	3,511 ± 253	13,900 ± 510	262 ± 21	1,580 ± 80
(+)-Amphetamine	7.07 ± 0.95	38.9 ± 1.8	1,765 ± 94	3,830 ± 170	24.8 ± 3.5	34 ± 6
(−)-Methamphetamine	28.5 ± 2.5	234 ± 14	4,640 ± 243	14,000 ± 644	416 ± 20	4,840 ± 178
(+)-Methamphetamine	12.3 ± 0.7	48.0 ± 5.1	736 ± 45	2,137 ± 98	24.5 ± 2.1	114 ± 11
(+)-Fenfluramine	302 ± 20	1,290 ± 152	51.7 ± 6.1	150 ± 5	>10,000	22,000 ± 1,100
(±)-Fenfluramine	739 ± 57	1,987 ± 205	79.3 ± 11.5	269 ± 7	>10,000	23,700 ± 1,300
(−)-Ephedrine	72.4 ± 10.2	225 ± 36	>10,000	>50,000	1,350 ± 124	4,398 ± 213
Tyramine	40.6 ± 3.5	72.5 ± 5.0	2,775 ± 234	1,556 ± 95	119 ± 11	106 ± 6.0
(±)-MDMA	77.4 ± 3.4	462 ± 18	56.6 ± 2.1	238 ± 13	376 ± 16	1,572 ± 59
Norepinephrine	164 ± 13	63.9 ± 1.6	>10,000	>50,000	869 ± 51	357 ± 27
Dopamine	66.2 ± 5.4	40.3 ± 4.4	>10,000	6,489 ± 200	86.9 ± 9.7	38.3 ± 1.6
5HT	>10,000	3,013 ± 266	44.4 ± 5.3	16.7 ± 0.9	>10,000	2,703 ± 79
GBR12935	>10,000	277 ± 23	>10,000	289 ± 29	>10,000	3.70 ± 0.40
GBR12909	>10,000	79.2 ± 4.9	>10,000	73.2 ± 1.5[1]	>10,000	4.3 ± 0.3[1]
Cocaine	>10,000	779 ± 30	>10,000	304 ± 10[2]	>10,000	478 ± 25[2]
Mazindol	>10,000	2.88 ± 0.17	>10,000	272 ± 11	>10,000	25.9 ± 0.56
Desipramine	>10,000	8.32 ± 1.19	>10,000	350 ± 13	>10,000	5,946 ± 193
Fluoxetine	>10,000	688 ± 39	>10,000	9.58 ± 0.88	>10,000	>5,000[1]
Citalopram	>10,000	4,332 ± 295	>10,000	2.40 ± 0.09	>10,000	20,485 ± 923
RTI-55	>10,000	5.89 ± 0.53	>10,000	1.00 ± 0.03	>10,000	0.83 ± 0.09
RTI-229	>10,000	19.5 ± 0.6	>10,000	362 ± 13	>10,000	2.15 ± 0.24
Indatraline	>10,000	12.6 ± 0.5	>10,000	3.10 ± 0.09	2,810 ± 777	1.90 ± 0.05

Each value is the mean ± SD of three experiments.
[1]Data from (Rothman et al., 1993).
[2]Data from Matecka et al. (1996).

(IC_{50} > 10,000 nM). Tyramine had a profile similar to that of (+)-METH, but was less potent: NE release (IC_{50} = 40.6 nM), DA release (IC_{50} = 119 nM) and 5-HT release (IC_{50} = 2,775 nM). The neurotoxic amphetamine analog, MDMA, was essentially equipotent at 5-HT and NE release (IC_{50} values = 56.6 and 77.4 nM, respectively) and considerably weaker at DA release (IC_{50} = 376 nM).

The release assays were also used to profile stimulant medications developed in the early 1960s. Phentermine had an amphetamine-type profile, being most potent at NE release (IC_{50} = 39.4 nM) followed by DA release ($IC50$ = 262 nM) and then 5-HT release (IC_{50} = 3,511 nM). Aminorex was almost equipotent at NE (IC_{50} = 26.4 nM) and DA (IC_{50} = 49.4 nM) and more potent at 5-HT release (IC_{50} = 193 nM) than phentermine, (+)-METH, or (+)-amphetamine. Chlorphentermine was the most potent and selective 5-HT releaser: IC_{50} = 30.9 nM for 5-HT release, 2,650 nM for DA release, and >10,000 nM for NE release. Although ineffective at NE release, chlorphentermine was a moderately potent NE uptake inhibitor (IC_{50} = 338 nM), indicating that a single agent may be a substrate at one transporter and an uptake inhibitor at another. (+)-Fenfluramine potently released 5-HT (IC_{50} = 51.7 nM) and less potently released NE (IC_{50} = 302 nM). (+)-Fenfluramine was essentially inactive at DA uptake or release.

DISCUSSION

Mechanism of action of stimulants

Using the release assay, it was possible to characterize the mechanism of action of stimulants which have received scant contemporary attention: ephedrine, aminorex, phentermine, and chlorphentermine.

In a previous study we used a different method, described in detail in that publication, to classify a drug as a substrate or uptake inhibitor (Rothman et al., 1999). Aminorex was classified as an uptake inhibitor at the DA transporter and a 5-HT transporter substrate. Phentermine was classified as DA transporter substrate and as a weak substrate or an uptake inhibitor at the 5-HT transporter. This method classified chlorphentermine as a 5-HT transporter substrate and DA uptake inhibitor. The more refined methods used here, which directly determine substrate-type activity, yielded different results: aminorex and chlorphentermine are DA transporter substrates and phentermine is a weak 5-HT transporter substrate. The core finding of our previous article (Rothman et al., 1999) that aminorex, (+)-fenfluramine, and chlorphentermine are 5-HT transporter substrates has been confirmed.

The low potency of (+)-METH and (+)-amphetamine at 5-HT release and uptake inhibition is similar to that reported by others (Eshleman et al., 1999), who characterized these agents in uptake inhibition assays using cloned DA, 5-HT, and NE transporters. The results for other compounds studied here are, in general, similar to those observed using the cloned transporters (Eshleman et al., 1999). DA is taken up by both dopaminergic and noradrenergic nerves with about equal potency. Consistent with these data, selective noradrenergic uptake inhibitors increase extracellular DA in the n. accumbens, ventral tegmental area, and frontal cortex of rats (Reith et al., 1997; Carboni et al., 1990; Yamamoto and Novotney, 1998).

Observations that blockade of NE transporters increases extracellular DA can explain why dopamine transporter knockout mice self-administer cocaine (Rocha et al., 1998) and demonstrate cocaine-conditioned place preference (Sora et al., 1998). Although these data suggest that the DA transporter is not critical for mediating cocaine reward, the data do not rule out a role for mesolimbic DA as a mediator of cocaine reward. The inability of cocaine to elevate extracellular DA in the striatum of DAT knockout mice (Rocha et al., 1998) is consistent with the lack of NE transporters in this brain region. Thus, in the absence of the DA transporter it is reasonable to assume that NE nerves would accumulate some of the extracellular DA in the n. accumbens. Since cocaine is a potent inhibitor of the NE transporter (Eshleman et al., 1999), administration of cocaine would inhibit DA transport into NE nerve terminals, thereby increasing extracellular DA and triggering cocaine reward.

Drug self-administration in animals is mediated by increases in mesolimbic synaptic DA (Wise, 1996). Thus, one might expect, as noted for DA uptake inhibitors (Ritz et al., 1987), that the rank order of potency of the stimulants studied here at the DA transporter should be the same as their potency as reinforcers. As reported by Griffiths and associates for a baboon self-administration model (Griffiths et al., 1979; Sannerud et al., 1989), the rank-order of potency for self-administration was amphetamine (24.8 nM) > phentermine (262 nM) = MDMA (376 nM) > ephedrine (1,350 nM) = chlorphentermine (2,650 nM) ⩾ fenfluramine was not self-administered (>10,000 nM). The values in parentheses are the IC_{50} values for stimulating [^{3}H]DA release. Thus, for these stimulants the rank order of potency for DA release (Table V) is similar to their rank order of potency in self-administration. Phentermine, which is 10-fold less potent than amphetamine in reinforcement, is 10-fold less potent than amphetamine in releasing [^{3}H]DA.

Relevance of findings to stimulant-induced subjective effects in humans

The role of DA in mediating reward/reinforcement behavior in animal models is well accepted and supported by the data. Although it is tempting to assume that the neurochemical mechanisms mediating reinforcement behavior in animal models of drug-seeking behavior and the neurochemical mechanisms mediating the subjective effects of stimulants in humans are the same, the neurochemical mediator of amphetamine-like positive subjective effects in humans remains to be established. Indeed, as noted in the Introduction, considerable data suggest that DA may be necessary, but not sufficient, to produce stimulant-induced subjective effects in humans (Rothman and Glowa, 1995; Rothman, 1994; Brauer et al., 1997; Villemagne et al., 1999; Ohuoha et al., 1997; Malison et al., 1997; Price et al., 1997; Brauer and de Wit, 1996, 1997). The authors wish to emphasize that we are not questioning the role of DA as a mediator of reinforcement behavior in animal models of drug-seeking behavior, but rather ask: What neurochemicals contribute to stimulant-induced subjective effects (the "high") in humans?

Among the substrate-type stimulants tested here, phentermine, MDMA, amphetamine, (+)-METH, and ephedrine produce amphetamine-like subjective effects in humans (Griffiths et al., 1979; Vollenweider et al., 1998; Brauer et al., 1996; Chait, 1994). Aminorex acts as a locomotor stimulant in animals and generalizes to the discriminative cue of amphetamine, and would be expected to produce amphetamine-like subjective effects in humans (Woolverton et al., 1994). Fenfluramine (Griffith et al., 1975) and chlorphentermine (Griffith et al., 1976) do not produce amphetamine-like subjective effects in humans. Our results show that the most potent action of the stimulants which produce amphetamine-like subjective effects is to release NE. Importantly, (+)-amphetamine, (−)-ephedrine, phentermine, and MDMA are 3.5-fold, 19-fold, 6.6-fold, and 4.8-fold more potent at NE release than DA release. These data raise the possibility that the release of NE contributes to the positive subjective effects produced by these substrate-type stimulants. We term this the "noradrenergic hypothesis."

A direct prediction of the release data and the noradrenergic hypothesis is that oral doses of these medications will produce sympathomimetic effects, which are mediated via release of NE, and amphetamine-like subjective effects, at lower doses than effects which are mediated by DA release. In humans, the noradrenergic effects of these compounds can be assessed via measurement of physiological parameters such as systolic blood pressure. The dopaminergic effects of these compounds can be assessed by their effect on plasma prolactin levels, which are decreased by dopaminergic agonists (Ascoli and Segaloff, 1996), and apparently not affected by agents which increase NE, such as the selective NE uptake inhibitor maprotiline (Steiger et al., 1993) or the selective NE releaser and adrenergic agonist ephedrine (Angrist et al., 1977).

The noradrenergic hypothesis receives strong support from studies conducted in humans. Oral doses of D-amphetamine in the range of 30–40 mg produce sympathomimetic effects and subjective effects with the same time course (Heishman and Henningfield, 1991; Martin et al., 1971). Importantly, D-amphetamine at this dose range does not decrease plasma prolactin (Grady et al., 1996; Mas et al., 1999), which should occur if D-amphetamine were releasing DA. Similar findings are reported for METH (Martin et al., 1971; Gouzoulis-Mayfrank et al., 1999). Moreover, MDMA-induced subjective effects occur at the same doses and with the same time course as its sympatho-

mimetic effects (Vollenweider et al., 1998; Mas et al., 1999). Since MDMA releases NE and 5-HT with the same potency, our data predict that MDMA should release prolactin, a serotonergic effect (Coccaro et al., 1996), at the same dose range and time course as its sympathomimetic effects. Indeed, MDMA increased plasma prolactin with the same time course as its sympathomimetic effects (Mas et al., 1999). As noted above, ephedrine-induced subjective effects occur at the same doses and with the same time course as its sympathomimetic effects (Griffiths et al., 1979) and ephedrine does not decrease prolactin (Angrist et al., 1977). Comparable oral doses of phentermine and amphetamine produce similar subjective effects, yet, as noted above, phentermine is 10-fold weaker at DA release than amphetamine, suggesting that release of DA does not mediate phentermine-induced subjective effects. Consistent with the noradrenergic hypothesis, oral doses of stimulants which produce subjective effects are correlated with their potency in releasing NE, not DA (Fig. 4). These data, summarized in Table VI, suggest that these medications produce amphetamine-like subjective effects at doses which activate the noradrenergic, not the dopaminergic system.

Orally administered dextroamphetamine (5–10 mg two or three times per day) is widely used to treat attention deficit disorder (Elia et al., 1999). As noted above, amphetamine probably does not release DA at this dose range. Thus, our results suggest that the primary neurochemical mechanism for the efficacy of amphetamine in treating attention deficit disorder results from release of NE, perhaps leading to activation of central alpha$_2$ adrenergic receptors. Consistent with this notion, direct alpha$_2$ agonists, such as clonidine and guanafacine, effectively treat attention deficit disorder (Hunt et al., 1986, 1995).

Medications whose primary mechanism of action is inhibition of NE reuptake (antidepressants) do not produce amphetamine-like subjective effects. Uptake inhibitors activate negative feedback loops which decrease cell firing rates via somatodendritic autoreceptors. This is observed for the dopaminergic, serotonergic, and noradrenergic systems (Diana et al., 1991; Moret and Briley, 1997; Mongeau et al., 1998; Cunningham and Lakoski, 1990). Transporter substrates, however, release neurotransmitter in a nerve-impulse independent manner and are not subject to these negative feedback mechanisms. Since the ability of uptake inhibitors to increase extracellular neurotransmitter is nerve impulse-dependent (Hurd and Ungerstedt, 1989b), reuptake inhibitors may not elevate extracellular neurotransmitter to the level achieved by transporter substrates. This may explain why NE uptake inhibitors do not produce amphetamine-like subjective effects.

Cocaine blocks the reuptake of NE, DA, and 5-HT with similar potency (Table V). As reviewed in the

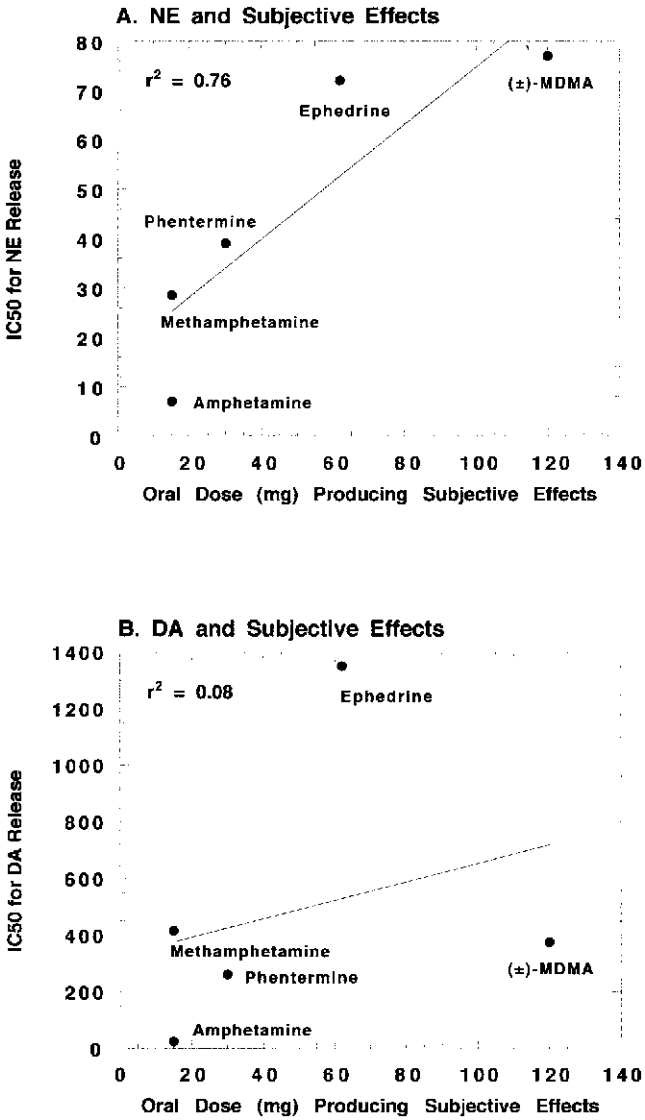


Fig. 4. Correlation of oral doses of stimulants which produce amphetamine-like subjective effects with their potency in releasing [^{3}H]NE (**A**) and [^{3}H]DA (**B**).

Introduction, a growing body of data suggests that DA is not the sole mediator of cocaine subjective effects in humans. Unlike antidepressants, cocaine produces intense amphetamine-like subjective effects. Assuming for the moment that this is due to increases in extracellular NE, the fundamental difference between cocaine and antidepressants may be the rate at which cocaine enters the brain (Balster and Schuster, 1973). Alternatively, cocaine binding sites not associated with the biogenic amine transporters might contribute to cocaine-induced subjective effects (Rothman et al., 1998b).

Although it is tempting to speculate that stimulant-induced positive subjective effects in humans are mediated by a single neurotransmitter, the more likely scenario is that multiple neurochemicals and brain

TABLE VI. Summary of the noradrenergic hypothesis

Drug	Oral dose producing amphetamine-type subjective effects	Oral dose producing sympathomimetic effects (NE-mediated)	IC_{50} for NE release	IC_{50} for DA release	Decrease plasma prolactin? (DA-mediated)
Amphetamine					
Reference 1	34 mg	34 mg			No
Reference 2	40 mg	40 mg	7	25	No
Reference 3	30 mg	30 mg			No
Phentermine					
Reference 4	30 mg	30 mg	39	262	Not reported
Ephedrine					
Reference 5	75 mg	75 mg	72	1,350	Not reported
Reference 6	35–50 mg	35–50 mg			No
MDMA					
Reference 7	120	120	77	376	No
Methamphetamine					
Reference 8	15 mg	15 mg			Not reported
Reference 9	14 mg	14 mg	28.5	416	No

Data supporting the hypothesis that oral doses of stimulants produce amphetamine-type subjective effects without releasing DA are summarized above. References: 1: Grady et al. (1996); 2: Mas et al. (1999); 3: Dommisse et al. (1984); 4: Brauer et al. (1996); 5: Martin et al. (1971); 6: Angrist et al. (1977); 7: Mas et al. (1999); 8: Martin et al. (1971); 9: Gouzoulis-Mayfrank et al. (1999).

regions contribute to the subjective experience described as the "high." The finding that low doses of selective NE, DA, and 5-HT uptake inhibitors enhance the cocaine discriminative cue in rats may support this notion (Cunningham and Callahan, 1991). Clearly, additional experiments are indicated to test the "noradrenergic hypothesis." In particular, it would be of interest to develop a highly selective NE releaser and determine its subjective effects in humans. These findings underscore the importance, in developing potential treatment agents for (+)-METH abuse, of neutralizing the effects of (+)-METH on the NE system (Rothman et al., 2000). These findings emphasize, moreover, that stimulant-induced reinforcement behavior in animals and stimulant-induced positive subjective effects in humans may utilize different neurochemical systems. The possibility that NE contributes to stimulant-induced positive subjective effects in humans does not rule out a role for DA in either subjective effects or stimulant addiction. For example, it is possible that even if a "high" is mediated mostly by NE, the intense repetitive drug-taking behavior seen in severely addicted individuals is mediated by mesolimbic DA. Perhaps most importantly, our data highlight the importance of considering the actions of cocaine and stimulants on neuronal systems other than DA, both to understand their mechanism of action and to develop effective pharmacotherapeutic medications (Rothman et al., 1998a; Baumann and Rothman, 1998).

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