In Vitro Characterization of Ephedrine-Related Stereoisomers at Biogenic Amine Transporters and the Receptorome Reveals Selective Actions as Norepinephrine Transporter Substrates

RICHARD B. ROTHMAN, NGA VU, JOHN S. PARTILLA, BRYAN L. ROTH, SANDRA J. HUFEISEN, BETH A. COMPTON-TOTH, JON BIRKES, RICHARD YOUNG, and RICHARD A. GLENNON

Clinical Psychopharmacology Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland (R.B.R., N.V., J.S.P.); Departments of Biochemistry, Neuroscience, and Psychiatry and the National Institutes of Mental Health Psychoactive Drug Screening Program, Case Western Reserve University School of Medicine, Cleveland, Ohio (B.L.R., S.J.H., B.A.C.-T., J.B.); and Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, Virginia (R.Y., R.A.G.)

ABSTRACT

Ephedrine is a long-studied stimulant available both as a prescription and over-the-counter medication, as well as an ingredient in widely marketed herbal preparations, and is also used as a precursor for the illicit synthesis of methamphetamine. Ephedrine is related to phenylpropanolamine, a decongestant removed from the market place due to concerns that its use increased the risk of hemorrhagic stroke. Standard pharmacology texts emphasize that ephedrine is both a direct and indirect adrenergic agonist, activating adrenergic receptors both by direct agonist activity as well as by releasing norepinephrine via a carrier-mediated exchange mechanism. Chemically, ephedrine possesses two chiral centers. In the present study, we characterized the stereoisomers of ephedrine and the closely related compounds pseudoephedrine, norephedrine, pseudonorephedrine (cathine), methcathinone, and cathinone at biogenic amine transporters and a large battery of cloned human receptors (e.g., "receptorome"). The most potent actions of ephedrine-type compounds were as substrates of the norepinephrine transporter (EC_{50} values of about 50 nM) followed by substrate activity at the dopamine transporter. Screening the receptorome demonstrated weak affinity at α_2-adrenergic and 5-hydroxytryptamine$_7$ receptors (K_i values 1–10 μM) and no significant activity at β-adrenergic or α_1-adrenergic receptors. Viewed collectively, these data indicate that the pharmacological effects of ephedrine-like phenylpropanolamines are likely mediated by norepinephrine release, and although sharing mechanistic similarities with, they differ in important respects from those of the phenylpropanonamines methcathinone and cathinone and the phenyisopropylamines methamphetamine and amphetamine.

The phenylpropanolamines represent a class of compounds that affect the sympathetic nervous system by a variety of mechanisms that include direct agonist activity at adrenergic receptors and "indirect" effects via carrier-mediated exchange with norepinephrine (NE) (Gilman et al., 1992). One of the best-known phenylpropanolamines is ephedrine. Ephedrine is a long-studied and widely used stimulant, available both as a prescription and an over-the-counter medication, as well as an ingredient in widely marketed herbal preparations. Current and past therapeutic applications of ephedrine include its use for asthma and its use as a hypertensive agent, decongestant, central stimulant, and anorectic agent (Kalix, 1991). Ephedrine has also attracted unfavor-

able attention because of its use as a synthetic precursor in the illegal production of methamphetamine (2003) and for its potential for toxicity (including death) (Haller and Benowitz, 2000).

Ephedrine possesses two chiral centers. Thus, ephedrine-related phenylpropanolamines can exist as four stereoisomers (Table 1). [1R,2S]-(−)-2-(Methylamino)-1-phenylpropan-1-ol is typically identified as (−)-ephedrine, and [1S,2R]-(+)-2-(methylamino)-1-phenylpropan-1-ol is typically identified as (+)-ephedrine. [1R,2R]-(−)-2-(Methylamino)-1-phenylpropan-1-ol is (−)-pseudoephedrine, and [1S,2S]-(+)-2-(methylamino)-1-phenylpropan-1-ol is (+)-pseudoephedrine. Removal of the methyl group from the nitrogen also results in four optical isomers: [1R,2S]-(−)-2-(amino)-1-phenylpropan-1-ol, commonly termed (−)-norephedrine or (−)-phenylpropanolamine; [1S,2R]-(−)-2-(amino)-1-phenylpropan-1-ol, commonly referred to as (+)-norephedrine or (+)-phenylpropanolamine; (−)-cathine or (−)-pseudonorephedrine;
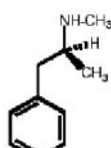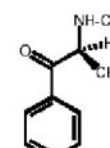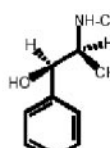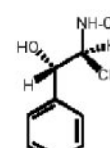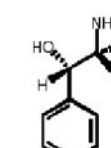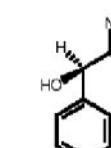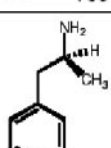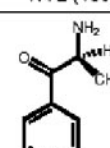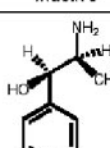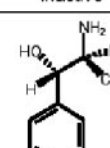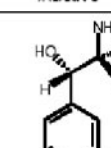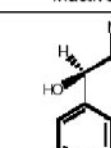
ABBREVIATIONS: NE, norepinephrine; PPA, phenylpropanolamine; NET, norepinephrine transporter; MPP$^+$, 1-methyl-4-phenylpyridinium; 5-HT, 5-hydroxytryptamine; DA, dopamine.

TABLE 1
Effect of test agents on biogenic amine release or uptake [EC$_{50}$ (nM $\pm$ S.D.)]^a

Effect of Test Agents on Biogenic Amine Release or Uptake [EC$_{50}$, ($\pm$SD)].^{a}

Phenylisopropylamines	Phenylpropanonamines	Phenylpropanolamines			
S(+)Meth-amphetamine	**S(−)Methcathinone**	**(−)Ephedrine**	**(+)Pseudoephedrine**	**(+)Ephedrine**	**(−)Pseudoephedrine**
NE 12.3^b	13.1 (0.6)	43.1 (4.0)	224 (14)	218 (14)	4092 (432)
DA 24.5^b	14.8 (0.4)	236 (9)	1988 (50)	2104 (68)	9125 (480) ^{c}
5-HT 736^b	1772 (160)	Inactive	Inactive	Inactive	Inactive
S(+)Amphetamine	**S(−)Cathinone**	**(−)Norephedrine**	**(+)Pseudonor-ephedrine**	**(+)Norephedrine**	**(−)Pseudo-norephedrine**
NE 7.1^b	12.4 (0.7)	42.1 (4.3)	15.0 (4.7)	137 (8.9)	30.1 (2.2)
DA 24.8^b	18.5 (0.3)	302 (10)	68.3 (2.4)	1371 (50)	294 (8)
5-HT 1765^b	2366 (138)	Inactive	Inactive	Inactive	Inactive

^{a} All agents behaved as substrates except for (−)-pseudoephedrine in the DA uptake assay.
^{b} Data reported previously (Rothman et al, 2001). These experiments used [^{3}H]DA and [^{3}H]NE instead of [^{3}H]MPP$^+$.
^{c} (−)-Pseudoephedrine behaved as an uptake inhibitor. Each value is the mean $\pm$ S.D. of three experiments.

and (+)-cathine or (+)-pseudonorephedrine. Table 1 shows how the structures of the phenylpropanolamines can be related to those of the phenylisopropylamines methamphetamine and amphetamine and the phenylpropanonamines methcathinone and cathinone, which differ only with respect to stereochemistry, and the oxidation state of the benzylic position. Methamphetamine and amphetamine lack a benzylic substituent, and S-(−)-methcathinone and S-(−)-cathinone can be viewed as analogs of ephedrine and norephedrine where the benzylic hydroxyl group has been oxidized to the corresponding ketone.

Although extensively studied for decades, to our knowledge the pharmacology of ephedrine-related phenylpropanolamines across a wide array of central nervous system receptors and the biogenic amine transporters has not been reported. Such information is important because of recent concerns regarding the risk of hemorrhagic stroke related to the use of phenylpropanolamine (PPA). This concern (Kernan et al., 2000) resulted in the removal of PPA from the U.S. market at the urging of the Food and Drug Administration. Whether other ephedrine-like compounds have a similar risk

is unknown, because the mechanism responsible for the increased risk of hemorrhagic stroke among users of PPA is unknown. Furthermore, many ephedrine-containing herbal preparations contain naturally occurring ephedra, and ephedra is known to possess other phenylpropanolamines in addition to ephedrine. Thus, in the present study we surveyed the interaction of these agents with a large battery of cloned human receptors (henceforth referred to as the receptorome; Setola et al., 2003; Sheffler and Roth, 2003), characterized their interactions in detail with the biogenic amine transporters, and compared them with their corresponding phenylpropanonamine and phenylisopropylamine counterparts. Because (−)-ephedrine has been used as a training drug in drug discrimination studies, we also sought to determine whether stimulus generalization potency of the phenylpropanolamines was related to their ability to bind at a particular population of receptors or to interact at a biogenic amine transporter. Finally, a further goal of this work was to formulate structure-activity relationships for the actions of the phenylpropanolamines at any sites at which they might interact. We discovered, to our surprise, that ephedrine and its

derivatives are relatively selective norepinephrine transporter (NET) substrates, suggesting that the pharmacological actions of these compounds result primarily from release of NE rather than direct activation of adrenergic receptors.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), weighing 300 to 400 g, were used as subjects in these experiments. Rats were housed in standard conditions (lights on from 7:00 AM to 7:00 PM) with food and water freely available. Animals were maintained in facilities fully accredited by the American Association of the Accreditation of Laboratory Animal Care, and experiments were performed in accordance with the Institutional Care and Use Committee of the National Institute on Drug Abuse, Intramural Research Program.

Drugs and Reagents. The phenylpropanolamine test drugs used in this study, S-(−)-methcathinone and S-(−)-cathinone (as their hydrochloride salts), were synthesized or obtained as described previously (Young and Glennon, 1998; Young et al., 1999). 1-(2-Diphenylmethoxyethyl)-4-(3-phenylpropyl)piperazine (GBR12935) was purchased from Sigma/RBI (Natick, MA). [^{3}H]MPP$^+$ (specific activity = 85 Ci/mmol) and [^{3}H]5-HT (specific activity = 27.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). The sources of other reagents are published in Rothman et al. (2001).

Radioligand Binding and Functional Assays. Stably and transiently transfected cells expressing mainly human cloned G-protein coupled receptors, ion channels, and transporters were maintained as detailed previously (Rothman et al., 2000; Roth et al., 2002; Setola et al., 2003) with radioligand binding and functional assays performed as described previously using the resources of the National Institute of Mental Health Psychoactive Drug Screening Program (Rothman et al., 2000; Roth et al., 2002). Details of all assay conditions have been published previously (Shi et al., 2003). Initial screening of test compounds was performed in quadruplicate at 10 μM concentration. For compounds that, on average, gave more than 50% inhibition, K_i values were obtained using 6 to 10 concentrations of unlabeled ligands as detailed previously (Rothman et al., 2000; Roth et al., 2002) with K_i values calculated using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, CA).

DA, NE, and 5-HT Release Assays. Our original method used [^{3}H]NE and [^{3}H]DA to measure release from noradrenergic and dopaminergic nerve terminals, respectively. Subsequently, we switched to using [^{3}H]MPP$^+$ as the radioligand for both the DA and NE release assays (Scholze et al., 2000) because this method led to an improved signal-to-noise ratio. Rat caudate (for DA release) or whole brain minus cerebellum and caudate (for NE and 5-HT release) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. Nomifensine (100 nM) and GBR12935 (100 nM) were added to the sucrose solution for [^{3}H]5-HT release experiments to block any potential [^{3}H]5-HT reuptake into NE and DA nerve terminals. For the DA release assay, 100 nM desipramine and 100 nM citalopram were added to block [^{3}H]MPP$^+$ uptake into NE and 5-HT nerves. For the NE release assay, 50 nM GBR12935 and 100 nM citalopram were added to block [^{3}H]MPP$^+$ uptake into DA and 5-HT nerves. After 12 strokes with a Potter-Elvehjem homogenizer, homogenates were centrifuged at 1,000g for 10 min at 0–4°C, and the supernatants were retained on ice (synaptosomal preparation).

Synaptosomal preparations were incubated to steady state with 5 nM [^{3}H]MPP$^+$ (60 min) or 5 nM [^{3}H]5-HT (60 min) in Krebs-phosphate buffer [without bovine serum albumin (BSA)] (pH 7.4) that contained 154.4 mM NaCl, 2.9 mM KCl, 1.1 mM CaCl$_2$, 0.83 mM MgCl$_2$, 5 mM glucose, 1 mg/ml ascorbic acid, and 50 μM pargyline plus 1 μM reserpine in a polypropylene beaker with stirring at 25°C. with the appropriate blockers. After incubation to steady state, 850 μl of synaptosomes preloaded with ^{3}H ligand was added to 12 × 75-mm polystyrene test tubes that contained 150 μl of test drug in

uptake buffer plus 1 mg/ml BSA. After 5 min ([^{3}H]5-HT) or 30 min (NE and DA assays), the release reaction was terminated by dilution with 4 ml of wash buffer (10 mM Tris-HCl, pH 7.4, containing 0.9% NaCl at 25°C) followed by rapid vacuum filtration over GF/B filters (Whatman, Maidstone, UK) using a harvester (Brandel, Inc., Gaithersburg, MD). The filters were rinsed twice with 4 ml of wash buffer using the harvester, and the retained tritium was counted by a liquid scintillation counter (Taurus; Titertek, Huntsville, AL) at 40% efficiency after an overnight extraction in 3 ml Cytoscint (ICN Biomedicals Inc., Costa Mesa, CA).

Substrate Reversal Experiments. In the substrate reversal experiments, test drugs were tested at approximately ED$_{70}$ doses in the absence and presence of blockers (250 nM GBR1209, 166 nM desipramine, and 100 nM fluoxetine) chosen to block the DA, 5-HT, and NE transporters, respectively. Substrate activity was detected by a significant reversal of the releasing effect of the test drug.

DA, 5-HT, and NE Uptake Assays. The effects of test agents on [^{3}H]DA, [^{3}H]5-HT, and [^{3}H]NE uptake were evaluated using published methods (Rothman et al., 2001). Briefly, synaptosomes were prepared from rat caudate for [^{3}H]DA reuptake, or from whole brain minus caudate and cerebellum for [^{3}H]5-HT and [^{3}H]NE reuptake. Fresh tissue was homogenized in ice-cold 10% sucrose using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1,000g for 10 min at 4°C, and supernatants were retained on ice (synaptosomal preparation). Polystyrene test tubes (12 × 75 mm) received 50 μl of Krebs-phosphate buffer (final pH 7.4) consisting of 0.5 mM Na$_2$SO$_4$, 0.5 mM KH$_2$PO$_4$, 126 mM NaCl, 2.4 mM KCl, 0.83 mM CaCl$_2$, 0.8 mM MgCl$_2$, and 11.1 mM glucose at pH 7.4, with 1 mg/ml ascorbic acid, 1 mg/ml BSA, and 50 μM pargyline added (uptake buffer). Subsequently, 750 μl of [^{3}H]DA (5 nM), [^{3}H]5-HT (2 nM), or [^{3}H]NE (5 nM) diluted in uptake buffer without BSA, and 100 μl of test agent in uptake buffer, were added to the tubes. Nonspecific uptake was defined using 10 μM tyramine for [^{3}H]DA and [^{3}H]NE assays, or 100 μM tyramine for [^{3}H]5-HT assays.

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–100 μM final tube concentration) diluted in uptake buffer. [^{3}H]5-HT uptake was conducted in the presence of 100 nM nomifensine and 100 nM GBR12935 to prevent uptake of [^{3}H]5-HT into NE or DA nerve terminals. [^{3}H]NE uptake was conducted in the presence of 5 nM RTI-229 to prevent uptake of [^{3}H]NE into DA nerve terminals. Incubations were carried out at 25°C for a periods of 10, 15, and 30 min for [^{3}H]NE, [^{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 GF/B filters and two additional wash cycles. The tritium retained on the filters was counted in a beta counter (Taurus; Titertek) at 40% efficiency after an overnight extraction into ICN Cytoscint cocktail (ICN Biomedicals Inc.).

Data Analysis and Statistics. As described previously (Rothman et al., 1993), IC$_{50}$ and EC$_{50}$ values for transporter assays were determined using the nonlinear least-squares curve fitting program MLAB-PC (Civilized Software, Bethesda, MD). In substrate reversal experiments, statistical significance was determined using the Student's t test.

Results

Receptorome Screen. The results are reported visually in Fig. 1. Most agents showed significant activity at NE transporter binding and little activity at DA and 5-HT transporter binding (Tables 1 and 2). Interestingly, these agents had little activity at adrenergic receptors when screened at 10 μM final concentration, and only three agents demonstrated enough activity in the screen to warrant K_i determinations. (−)-Norephedrine had a K_i value of 5,000 ± 700 nM
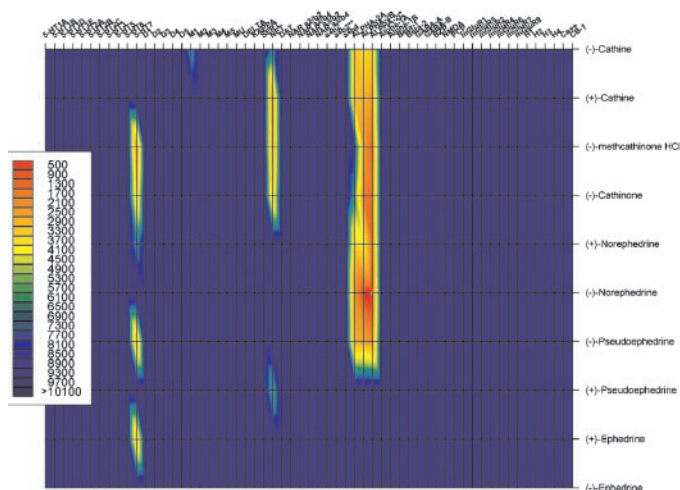
Fig. 1. Receptorome profile of ephedra analogs. Shown is a mesh plot of the mean K_i values (units are nanomolar concentration) for the test agents at a large number of cloned receptor, transporters, and ion channels (e.g., receptorome created using SigmaPlot version 8.0 as described elsewhere; Setola et al., 2003). K_i values are color-coded according to the inset.

at rat β_1-adrenergic receptors and was inactive at rat β_2-adrenergic receptors. (−)-Pseudoephedrine and (−)-methcathinone had a K_i values of 5,330 ± 510 and 2,930 ± 170 nM, respectively, at the human α_{1A} receptor. All agents were inactive at cholinergic, nicotinic, GABA, histaminergic, prostaglandin, opioid, N-methyl-D-aspartate receptors, dopamine, and most 5-HT receptors. Significant activity was observed at α_2-adrenergic receptors and the 5-HT$_7$ serotonin receptor, although the K_i values were typically in the low micromolar range (Table 2). Because phenylpropanolamines are thought to have direct agonist activity at α_1-adrenergic receptors, all agents were tested for functional activity at the human α_{1A} and α_{2A} receptors. All agents were inactive (Table 3). To our knowledge, this represents the first reported comprehensive pharmacological screen of these commonly used drugs.

Transporter Activity. The agents were first screened for activity in the uptake and release assays at a single 10 μM concentration. Compounds producing greater than 50% inhibition were further characterized with dose-response curves. Agents demonstrating activity in the uptake inhibition assay and not the release assay were classified as uptake inhibitors. Only (−)-pseudoephedrine (Table 1) acted as uptake inhibitor. Methylphenidate, used as a positive control, was a potent inhibitor of DA and NE uptake (IC$_{50}$ values = 90.2 ± 7.9 and 118 ± 12 nM, respectively). (−)-Pseudoephedrine, on the other hand, was a weak (IC$_{50}$ = 9125 nM) DA uptake inhibitor. All the other agents were substrates. EC$_{50}$ values are reported in Table 1. Substrate activity was confirmed via substrate-reversal experiments. Figure 2 illustrates the reversal of DA release induced by putative DA transporter substrates by GBR12909. Similar results were obtained for NE release (data not shown).

In an attempt to determine whether a relationship exists between the ability of the examined agents to release NE and their potency to substitute for (−)-ephedrine in drug discrimination studies, correlation studies were performed using previously published data. Animals (rats) were trained to discriminate (−)-ephedrine from saline vehicle and tests of

stimulus generalization were conducted with each of the phenylpropanolamine isomers, (+)-amphetamine, and S-(−)-methcathinone (Young and Glennon, 1998; Young et al., 1999). Only two phenylpropanolamines failed to substitute for (−)-ephedrine: (−)-pseudonorephedrine and (−)-pseudoephedrine. (−)-Pseudonorephedrine disrupted the animals behavior at low doses and further substitution tests were precluded; in contrast, (−)-pseudoephedrine was essentially inactive up to doses of 12 mg/kg (Young et al., 1999). Both S-(+)-amphetamine and S-(−)-methcathinone also substituted for the (−)-ephedrine stimulus. The (−)-ephedrine stimulus generalized to (−)-methamphetamine; but (+)-methamphetamine, like (−)-pseudonorephedrine, disrupted the animals' behavior. Examining all agents that substituted for (−)-ephedrine, a correlation coefficient (r) of 0.711 was obtained; however, if (+)-pseudonorephedrine is removed from the analysis, the r value is 0.924 (Fig. 3B). This indicates that the activity of (+)-pseudonorephedrine should probably not be explained solely in terms of its ability to release NE. Similar correlations were obtained when substitution potency was plotted against the ability of the agents to release DA (not shown). However, because NE and DA releasing potency are intercorrelated (Fig. 3A), the significance of these correlations is unknown and may simply be fortuitous.

Discussion

The major finding of the present study is that the most potent action of ephedrine-like phenylpropanolamines is substrate activity at NE transporters. These compounds have much lower and/or negligible affinity for adrenergic receptors, indicating that the pharmacological effects of ephedrine-like phenylpropalolamines most likely occurs via the indirect release of NE.

Structure-Activity Considerations

The availability of neurotransmitter release data allows for the first time a structure-activity assessment among the phenylpropanolamine isomers, and a direct comparison with their oxidized counterparts.

NE Release. In the N-methyl series, introduction of a benzylic carbonyl oxygen atom has no effect on activity comparing S-(+)-methamphetamine with S-(−)-methcathinone. Reduction of the carbonyl group to an alcohol reduces activity by about 3-fold in going from methcathinone to (−)-ephedrine, but reduces activity slightly more when stereochemistry about the benzylic atom is reversed [i.e., (+)-pseudoephedrine]. Reversing the stereochemistry about the α-position of (−)-ephedrine reduces activity by nearly 100-fold, whereas the same change in (+)-pseudoephedrine has no effect on activity [i.e., (+)-ephedrine]. In the primary amine series, introduction of the carbonyl oxygen atom (i.e., cathinone) and its reduction to an alcohol [i.e., (−)-norephedrine] has an effect similar to what was seen in the N-methyl series. Interestingly, however, reversal of stereochemistry about the benzylic position results in a severalfold enhancement of activity [i.e., (+)-pseudonorephedrine] rather than the severalfold decrease in affinity seen in the N-methyl series. Also, in contrast to the N-methyl series, reversal of stereochemistry about the α-position of (−)-norephedrine resulted in little effect [i.e., (−)-pseudonorephedrine] rather than a 100-fold

TABLE 2

Effect of test agents on α-adrenergic and 5-HT$_7$ receptors (K_i, nM $\pm$ S.D.)

K_i values for the α_2-adrenergic and 5-HT$_7$ receptors. Empty cells lack values because the compounds were inactive at those binding sites.

	α_{2A}	α_{2B}	α_{2C}	5-HT$_7$
(−)-Ephedrine		648 ± 84	708 ± 100	
(+)-Ephedrine				2,300 ± 900
(−)-Pseudoephedrine	2,011 ± 571	1,870 ± 175	1,220 ± 164	2,000 ± 600
(−)-Norephedrine	1,330 ± 207	400 ± 84	265 ± 19	
(+)-Norephedrine	3,024 ± 631	3,940 ± 280	597 ± 170	7,000 ± 2,400
(−)-Cathinone	6,808 ± 1,555	918 ± 234	765 ± 144	2,700 ± 650
(−)-Methcathinone	9,017 ± 744	1542 ± 256	605 ± 113	1,000 ± 220
(+)-Pseudonorephedrine ((+)-cathine)	2,608 ± 460	2,301 ± 126	1,547 ± 143	
(−)-Pseudonorephedrine [(−)-cathine]	2,598 ± 676	3,489 ± 335	2,804 ± 324	
Oxymetazoline	1.36 ± 0.11			
Prazosin		14.6 ± 3.7	14.5 ± 3.4	

TABLE 3

Functional activity at human α receptors

Stably expressing lines of human α_{1A}-adrenergic receptors (provided by Diane Perez, Cleveland Clinic, Cleveland, OH) and α_{2A}-adrenergic receptors (provided by Lee Limbird, Vanderbilt University, Nashville, TN) were used for functional studies. α_{1A}-Adrenergic receptor mediated activation of intracellular calcium mobilization was quantified using a FlexStation using the calcium flux assay kit (Molecular Devices Corp, Sunnyvale, CA) in the 96-well format. α_{2A}-Adrenergic receptor-expressing cells were transfected with $G\alpha16$ to provide coupling to phospholipase C and subsequent mobilization of intracellular calcium quantified using the calcium flux assay kit in the 96-well format (Pauwels and Colpaert, 2000).

	K_{act} (nM)	
Drug	α_{2A}	α_{1A}
(−)-Ephedrine	>10,000	>10,000
(+)-Ephedrine	>10,000	>10,000
(+)-Pseudoephedrine	>10,000	>10,000
(−)-Pseudoephedrine	>10,000	>10,000
(−)-Norephedrine	>10,000	>10,000
(+)-Norephedrine	>10,000	>10,000
(−)-Cathinone	>10,000	>10,000
(−)-Methcathinone	>10,000	>10,000
(+)-Pseudonorephedrine [(+)-cathine]	>10,000	>10,000
(−)-Pseudonorephedrine [(−)-cathine]	>10,000	>10,000
Epinephrine	N.D.	15 ± 1.7
Clonidine	3.7 ± 1.1	N.D.

N.D., not done.

decrease in activity, whereas this same structural modification of (+)-pseudonorephedrine reduced activity by 9-fold. Clearly, the presence of the N-methyl substituent can cause differences in potencies within the two series, and these differences are most evident with the 1S,2S and 1R,2R isomers (i.e., where the hydroxy and methyl substituents are on opposite faces of the molecule as drawn in Table 1) and result in unexpectedly higher activity for (+)-pseudonorephedrine and (−)-pseudonorephedrine.

DA Release. The same general trends seen with norepinephrine release were seen with dopamine release except that reduction of the carbonyl group of cathinone has a greater negative impact. That is, reduction of the carbonyl group of methcathinone and cathinone to (−)-ephedrine and (−)-norephedrine, respectively, decreased activity by about 15-fold. Also as was seen with norepinephrine release, the N-methyl substituents seem to play a role in activity and the 1S,2S and 1R,2R isomers (+)-pseudoephedrine and (−)-pseudonorephedrine were more potent than expected on the basis of the potencies of their N-methyl counterparts. In fact, (−)-pseudoephedrine behaved not as substrate but as uptake inhibitor of dopamine release.

Although the agents are generally more potent in releasing NE than DA, there is a significant correlation ($r = 0.964$; Fig. 3A) between the NE release and DA release potencies of the

agents examined. This suggests that the structural features controlling release of DA and NE among these agents are similar.

5-HT Release. The N-methyl analog methamphetamine is twice as potent as its primary amine counterpart amphetamine. Introduction of a benzylic oxygen atom in the form of a ketone further reduced activity by about 2-fold in both series. However, reduction of the ketone to an alcohol, as in the phenylpropanolamine derivatives, abolished activity regardless of stereochemistry.

Ephedrine-Like Agents

The ephedrine-like agents (−)-ephedrine, (+)-ephedrine, (−)-pseudoephedrine, (+)-pseudoephedrine, (−)-norephedrine, (+)-norephedrine, (−)-pseudonorephedrine [i.e., (−)-cathine], and (+)-pseudonorephedrine [i.e., (+)-cathine], were inactive at the 5-HT transporter. (−)-Ephedrine, along with the other ephedrine-like agents, was more potent at releasing NE than DA. Interestingly, in the present study, which used [^{3}H]MPP$^+$ to measure release from DA nerve terminals, the EC$_{50}$ value of (−)-ephedrine was 236 nM. In our previous study (Rothman et al., 2001), which used [^{3}H]DA, the EC$_{50}$ value was 1350 nM. The reason for this quantitative difference in potency using the different ligands is not apparent. (+)-Ephedrine, like (−)-ephedrine, was more potent at releasing NE than DA, although the EC$_{50}$ values were 5-fold and 9-fold lower than (−)-ephedrine at NE and DA release, respectively. The results obtained with (+)-pseudoephedrine were similar to those obtained for (+)-ephedrine. (−)-Pseudoephedrine was much weaker than (+)-ephedrine at NE release and was a very weak DA uptake blocker. This latter observation illustrates that a relatively slight structural modification can change a substrate to an uptake inhibitor. Similar results were observed previously for stereoisomers of phenmetrazine (Rothman et al., 2002). (−)-Norephedrine and (+)-norephedrine were similar to (−)-ephedrine and (+)-ephedrine, respectively.

Ephedrine is often described as an agent with both direct and indirect sympathomimetic activity. The term indirect refers to effects mediated by release of NE. "Direct" refers to effects mediated by direct agonist activation of adrenergic receptors. Before the advent of cellular expression systems using cloned receptors, agonist effects of ephedrine were determined using tissue systems that also possessed adrenergic nerve terminals. With these types of assays, it can be difficult to distinguish between direct agonist activation of adrenergic receptors by ephedrine and activation of adrenergic receptors
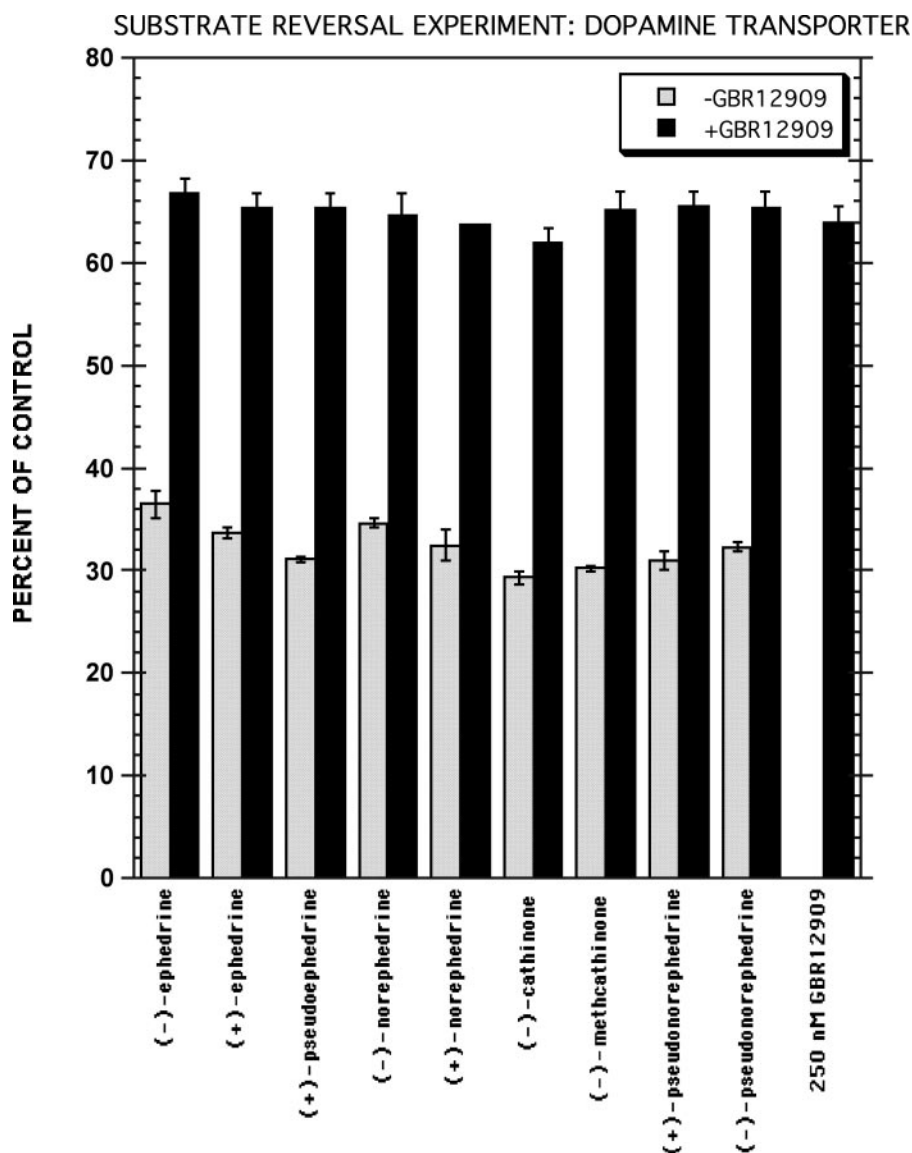
Fig. 2. Substrate reversal experiments for the DA transporter. Test drugs were tested at approximately EC_{70} doses in the absence and presence of 250 nM GBR1209, chosen to block the DA transporter. Substrate activity was detected by a significant reversal of the releasing effect of the test drug. Each value is the mean ($\pm$ S.D., $n = 3$). All changes were statistically significant ($p < 0.05$ compared with control, paired t test). The concentrations of test agents were as follows: ($-$)-ephedrine (475 nM), ($+$)-ephedrine (4,200 nM), ($+$)-pseudoephedrine (4,000 nM), ($-$)-norephedrine (600 nM), ($+$)-norephedrine (2,750 nM), (minus]-cathinone (40 nM), ($-$)-methcathinone (30 nM), ($+$)-pseudonorephedrine (140 nM), and ($-$)-pseudonorephedrine (600 nM).

mediated by ephedrine-stimulated NE release. For example, using reserpine to deplete endogenous NE, Kawasuji et al. (1996) reported that ($-$)-ephedrine has moderately potent direct agonist effects at β_1-adrenergic receptors. Consistent with this, Vansal and Feller (1999), using cloned human β_1-adrenergic receptors, reported a K_{act} value of 550 nM when these receptors were overexpressed. In contrast, we were unable to detect significant agonist actions at the cloned β-adrenergic receptors (data not shown). The EC_{50} value for NE release is 43 nM, an order of magnitude lower, suggesting that the most likely relevant pharmacological effect for ($-$)-ephedrine is NE release. Consistent with this hypothesis, it is noteworthy that all the agents tested in this study had negligible affinity for or agonist activity at cloned β-adrenergic and α_1-adrenergic receptors, suggesting, that like ($-$)-ephedrine, the most important pharmacological effect of these agents is likely to be NE release.

As noted in the Introduction, phenylpropanolamine was withdrawn from the marketplace because of concern that its use increased the risk of hemorrhagic stroke (Kernan et al., 2000). Our data indicate that ($-$)-phenylpropanolamine and ($+$)-phenylpropanolamine [($-$)-norephedrine and ($+$)-norephedrine) and ($-$)-ephedrine and ($+$)-ephedrine] have similar activity at the biogenic amine transporters. This suggests that the potential for hemorrhagic stroke may not arise as a direct result of the pharmacological mechanism of action, NE release, but rather from idiosyncratic vasculitis (Kase, 1986; Glick et al., 1987).

Cathinone-Like Agents

S-($-$)-Cathinone is a naturally occurring agent found in the Khat plant (*Catha edulis*) that exhibits amphetamine-like properties in animals and humans; ($+$)-pseudonorephed-rine or ($+$)-cathine, a phenylpropanolamine representing a
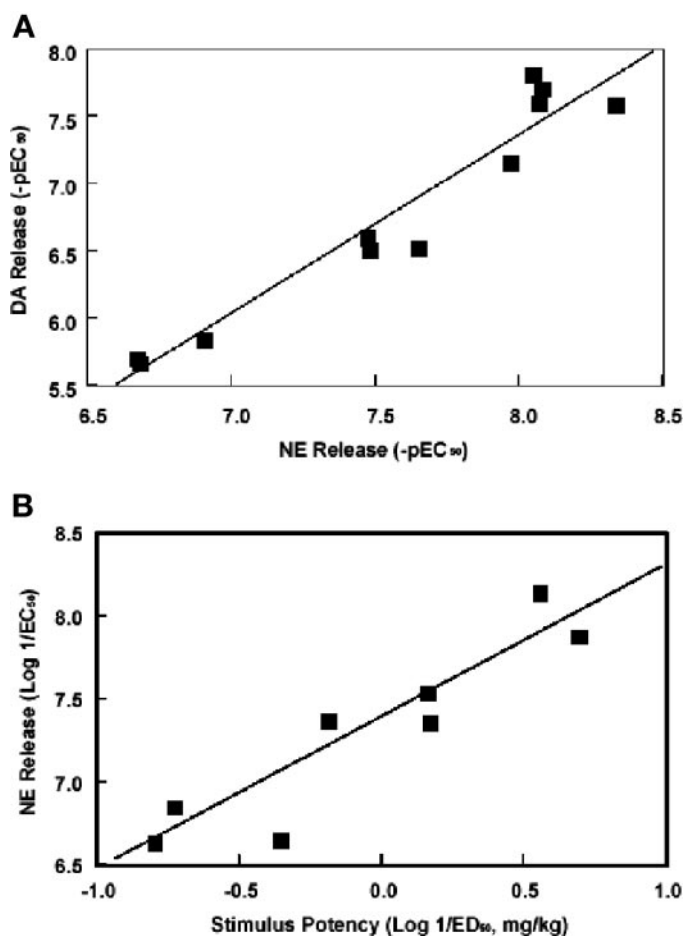
Fig. 3. Top, relationship between potency of agents on DA release versus NE release. $r = 0.964$ ($n = 11$). Bottom, stimulus generalization potencies (Young et al., 1999; Bondareva et al., 2002) of agents in $(-)$-ephedrine-trained animals versus their potency as NE-releasing agents. $r = 0.924$ ($n = 8$) [with $(+)$-pseudonorephedrine or $(+)$-cathine, $r = 0.711$].

reduced form of cathinone, retains weak stimulant character and is also found in the same plant (Zelger et al., 1980). S-$(-)$-Methcathinone is the N-methyl analog of S-$(-)$-cathinone; that is, from a structural perspective, methcathinone is to cathinone what methamphetamine is to amphetamine (Glennon et al., 1987). We examined S-$(-)$-cathinone and S-$(-)$-methcathinone for comparison with the phenylpropanolamines. As noted for the ephedrine-like agents, these compounds are weak or inactive at the 5-HT transporter. In contrast to the ephedrine-like agents, S-$(-)$-cathinone and S-$(-)$-methcathinone, like S-$(+)$-amphetamine (Rothman et al., 2001), have similar EC_{50} values for releasing NE and DA. $(+)$-Pseudonorephedrine [$(+)$-cathine] and to a greater extent $(-)$-pseudonorephedrine [$(-)$-cathine] loses activity at DA release. In general, with respect to 5-HT release, the phenylpropanonamines behave more like the phenylisopropylamines than like the phenylpropanolamines. However, the phenylisopropylamines additionally possess action as NE- and DA-releasing agents (Rothman et al., 2001). As observed with the ephedrine-like agents, the cathinone-like agents have micromolar K_i values for the α_2-adrenergic receptors. The physiological significance of the affinity of some of these agents for the 5-HT$_7$ receptors is not clear.

Summary

   The major finding of the present study is that the most potent action of ephedrine-like phenylpropanolamines is substrate activity at NE transporters. As a group, these compounds have much lower and/or negligible affinity for adrenergic receptors. Thus, the pharmacological effects of ephedrine-like phenylpropalolamines most likely occur via the indirect release of NE. The finding that the phenylpropanolamines act primarily via release of NE and that this is a property shared by amphetamine, methamphetamine, cathinone, and methcathinone, is consistent with an earlier proposal that ephedrine-like stimulus effects in rats are mediated primarily by NE release (Bondareva et al., 2002). Nevertheless, the ability of some of these agents to release DA, particularly amphetamine and methamphetamine (Rothman et al., 2001), might additionally contribute to their actions and might also account for behavioral differences, including the behavioral disruption noted with some of the agents as described above. The present study also demonstrates how relatively small structural changes (i.e., introduction and oxidation state of a benzylic oxygen atom, stereochemistry, and presence of an N-methyl group) can dramatically alter mechanistic possibilities for actions associated with these structurally simple phenylalkylamines.

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Address correspondence to: Dr. Richard B. Rothman, Clinical Psychopharmacology Section, Division of Intramural Research, National Institute on Drug Abuse, National Institutes of Health, 5600 Nathan Shock Dr., Baltimore, MD 21224. E-mail: rrothman@intra.nida.nih.gov