Therapeutic Potential of Monoamine Transporter Substrates

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Abstract: Monoamine transporter proteins are targets for many psychoactive compounds, including therapeutic and abused stimulant drugs. This paper reviews recent work from our laboratory investigating the interaction of stimulants with transporters in brain tissue. We illustrate how determining the precise mechanism of stimulant drug action (uptake inhibitor vs. substrate) can provide unique opportunities for medication discovery. An important lesson learned from this work is that drugs which display equipotent substrate activity at dopamine (DA) and serotonin (5-HT) transporters have minimal abuse liability and few stimulant side-effects, yet are able to suppress ongoing drug-seeking behavior. As a specific example, we describe the development of PAL-287 (α-methylnapthylethylamine), a dual DA/5-HT releasing agent that suppresses cocaine self-administration in rhesus monkeys, without the adverse effects associated with older phenylethylamine 5-HT releasers (e.g., fenfluramine) and DA releasers (e.g., amphetamine). Our findings demonstrate the feasibility of developing non-amphetamine releasing agents as potential treatments for substance abuse disorders and other psychiatric conditions.

Keywords: Transporters, dopamine, serotonin, release, amphetamine, PAL-287.

PSYCHOMOTOR STIMULANTS

Psychomotor stimulants are drugs that produce a spectrum of effects in humans characterized by cardiovascular activation, increased energy and mood elevation. After high doses or extended periods of use, stimulants can induce a range of disordered thought processes, including severe psychotic episodes. In laboratory animals, stimulants produce hyperactivity and support self-administration behavior, effects that are mediated by increases in extracellular dopamine (DA) in mesolimbic reward circuits (DA) [1]. The effects of stimulants are often described as "amphetamine-like" since amphetamine is the prototypical stimulant agent. Table **1** shows some examples of stimulants. Many of these drugs are medications with long histories of efficacy and safety, whereas others are highly addictive substances associated with significant morbidity and mortality. In some cases, as with amphetamine itself, the same drug can be therapeutic or abused depending upon the context in which the drug is administered. Indeed, the route of administration can play a crucial role in determining the addictive potential stimulants, since a slower rate of drug action is associated with diminished reinforcing strength [2, 3].

Most stimulants exert their pharmacological effects by interacting with monoamine transporter proteins expressed on various cell types. Nerve cells that synthesize, store, release and metabolize monoamine transmitters [DA, norepinephrine (NE) and serotonin (5-HT)] are widely distributed in the mammalian CNS. These cells display specialized membrane proteins that transport previously released transmitter from the extracellular space back into the cytoplasm [4, 5]. Distinct transporter proteins are expressed on NE neurons (i.e., NE transporters, NET), DA neurons (i.e., DA transporters, DAT), and 5-HT neurons (i.e., 5-HT transporters, SERT). These proteins belong to a superfamily of Na^+/Cl^- dependent transporters that share genetic, structural, and functional homologies [6, 7]. Under normal circumstances, the transporter-mediated uptake of amine transmitters is the principal mechanism for inactivation of monoamine signaling in the brain. Accordingly, stimulants that interact with monoamine transporters can have profound effects on normal neurotransmission [8]. It is important to note that certain stimulant drugs, especially substituted amphetamines, interact with other neuronal

Table 1. Representative Examples of Psychostimulants

Therapeutic Drugs	Indication
Methylphenidate	Attention Deficit Disorder
Amphetamine	Attention Deficit Disorder/Narcolepsy
Pemoline	Attention Deficit Disorder
Phentermine	Anorectic
Diethypropion	Anorectic
Phendimetrazine	Anorectic
Abused Drugs	
Cocaine	
Methamphetamine	
3,4-Methylenedioxymethamphetamine (MDMA)	
1-Benzylpiperazine (BZP)	

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proteins such as monoamine oxidase (MAO) [9] and the vesicular monoamine transporter type 2 ($VMAT_2$) [10].

Stimulants that target transporter proteins can be divided into two classes based on their precise molecular mechanism of action - reuptake inhibitors and substrate-type releasers. Reuptake inhibitors (e.g., methylphenidate and cocaine) bind to transporter proteins but are not transported. These drugs elevate extracellular transmitter concentrations by blocking transporter-mediated recapture of transmitters from the synapse. Substrate-type releasers (e.g., amphetamines) also bind to transporter proteins, and these drugs are subsequently transported into the neuronal cytoplasm where they evoke non-exocytotic transmitter release. Releasers elevate extracellular transmitter concentrations by a two-pronged mechanism: (1) they promote efflux of transmitter by reversing the normal direction of transporter flux and (2) they increase free cytoplasmic levels of transmitter by interacting with $VMAT_2$ to disrupt vesicular storage [11, 12]. The precise underpinnings of transporter-mediated release involve complex processes that are the topic of intensive investigation [13-15]. Because substrate-type releasing agents must be transported into nerve terminals to promote transmitter release, reuptake inhibitors can block the effects of releasers. In some cases, drugs can act as allosteric modulators of transporter function [16-20].

For more than a decade, we have carried out experiments investigating the interaction of psychostimulants with monoamine transporters in order to understand how these drugs affect the brain. It is anticipated that the knowledge gained from these studies will aid in the development of pharmacotherapies for treating stimulant dependence and other psychiatric disorders [21]. Specific aspects of our work have been recently reviewed [22-25]. In the present paper, we illustrate how determining the mechanism of action of a wide range of transporter ligands (uptake inhibitor vs. substrate) can provide unique opportunities for medication development.

TRANSPORTER MECHANISMS

As noted above, stimulants can be classified as either uptake inhibitors or releasers based on their molecular mechanism of action. Both types of agents increase synaptic concentrations of transmitter via transporter-dependent processes, and there are important differences in their modes of action at the cellular and organismic level. In particular, the activity of reuptake inhibitors requires ongoing release of transmitters via exocytosis – a cellular mechanism directly dependent upon electrical depolarization and extracellular calcium. Thus, the ability of reuptake inhibitors to increase synaptic transmitter levels is described as being impulse- and calcium-dependent. Substrates, on the other hand, increase synaptic transmitter levels by a process that is largely independent of ongoing cell firing and exocytotic transmitter release. Plasma membrane autoreceptors mediate negative feedback mechanisms that serve to dampen the ability of reuptake inhibitors to elevate synaptic transmitter [26]. Such negative feedback effects exist for 5-HT [27-29], DA [30], and NE [31] neuronal systems. While autoreceptor activation can abolish the ability of reuptake inhibitors to elevate synaptic transmitter levels, autoreceptor mechanisms have little or no effect on substrate-induced neurotransmitter release [32-36]. Because of autoreceptor-mediated feedback inhibition, reuptake inhibitors tend to produce small increases in extracellular neurotransmitter whereas releasers tend to produce more robust increases [37]. The *in vivo* microdialysis data in Fig. **1** illustrate the modest and sustained elevation of extracellular 5-HT evoked by the 5-HT reuptake inhibitor, fluoxetine, compared to the much larger and transient effect of the 5-HT releaser, (+)-fenfluramine [33, 38, 39].

Given the important distinctions between uptake inhibitors and releasers, we sought to develop assays which could easily determine if test drugs interacted with transporters as inhibitors or substrates. This work served to identify the mechanism of action of known stimulant-type agents [40-43] and to guide the synthesis and evaluation of novel treatment agents for addictive disorders [21]. Traditionally, it has been difficult to use simple test tube assays to discriminate between drugs that are uptake inhibitors vs. those that are substrate-type releasers [8, 11]. Thus we spent considerable time and effort in developing a rapid high-throughput method for measuring the release of preloaded [^{3}H]DA,

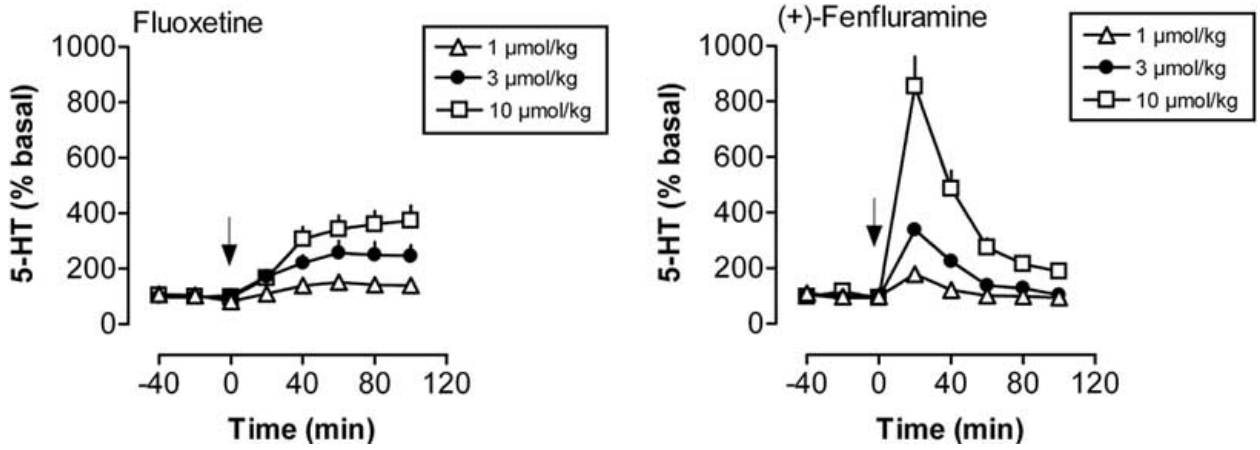


Fig. (1). Effects of fluoxetine (a 5-HT reuptake inhibitor) and (+)-fenfluramine (a 5-HT releaser) on extracellular 5-HT in rat nucleus accumbens. Dialysis methods were carried out as described previously [22]. Drugs were administered i.v. at time zero minutes. Data are expressed as a percentage of the mean of three basal dialysate samples collected prior to drug treatment. Basal dialysate 5-HT level was 0.46 ± 0.17 nM. Values are mean ± SEM for N=5 rats/group.

[^{3}H]NE and [^{3}H]5-HT from nervous tissue *in vitro* [44]. The basic strategy employed in the release assay is to first incubate synaptosomes with [^{3}H]neurotransmitter for sufficient time to achieve steady state. At steady state, test drugs are added to synaptosomes and the reaction is terminated by rapid filtration after 5 min. Transmitter "release" is quantified by measuring the amount of tritium retained on the filter – decreases in retained tritium reflect increases in [^{3}H]neurotransmitter released. A key requirement of our release assay is the inclusion of reserpine in the assay buffer; reserpine prevents accumulation of neurotransmitters into synaptic vesicles thereby maximizing the amount of preloaded [^{3}H]neurotransmitter available for substrate-induced release.

The activity of transporter substrates can be readily distinguished from that of transporter blockers using our *in vitro* release assay. As depicted in Fig. **2**, the non-selective uptake inhibitor indatraline, which has high affinity for DAT, NET and SERT, displays very weak activity in release assays. By contrast, the transporter substrate, methamphetamine, causes dose-dependent release of [^{3}H]DA, [^{3}H]NE and [^{3}H]5-HT. Methamphetamine is much more potent at releasing DA and NE when compared to its effects on 5-HT, consistent with the known pharmacology of this drug [40, 44]. The data in Fig. **3** demonstrate that low concentrations of indatraline antagonize methamphetamine-induced release of [^{3}H]DA, shifting the methamphetamine release curve to the right. Thus, indatraline blocks the DA-releasing activity of methamphetamine by binding to DAT sites in the tissue. The apparent K_i value for indatraline (~2 nM) calculated from the shift of the methamphetamine release curve is similar to its K_i value for inhibition of [^{3}H]DA uptake (1.9 nM). In more recent studies, we have employed [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) as a radiolabeled substrate for DAT and NET assays. [^{3}H]MPP$^+$ yields results comparable to the endogenous substrates DA and NE, with an improved signal-to-noise ratio [43]. The collective findings illustrate that our assay systems can readily discriminate releasers from uptake blockers. Moreover, the assays can be used to evaluate the pharmacological profile of test drugs at all three monoamine transporters under similar experimental conditions. Table **2** reports the results obtained with a wide range of pharmacological agents using these assays. Although the results are discussed in detail in the individual papers cited in Table **2**, we highlight important findings here.

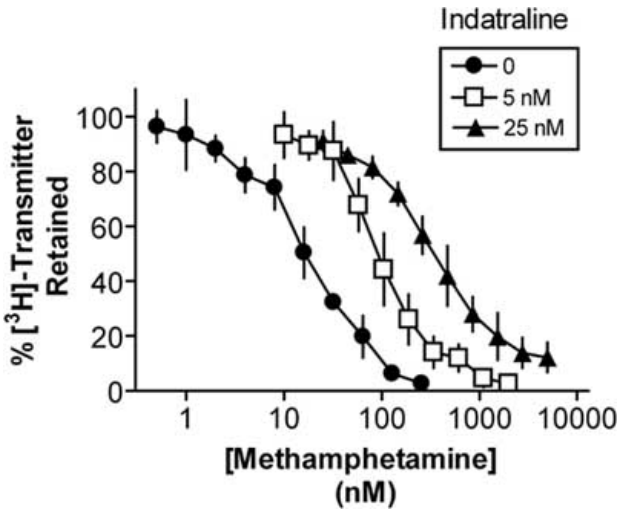


Fig. (3). Effects of indatraline (5 nM and 25 nM) on methamphetamine-evoked release of [^{3}H]dopamine. Each point is the mean±SD (n=3). Data are from [44].

APPETITE SUPPRESSANTS

Figure **4** shows that many clinically available appetite suppressants are phenylethylamines structurally related to amphetamine. Not surprisingly, these drugs share behavioral properties with abused stimulants but are typically less potent and less addictive [45-48]. Preclinical studies have shown that amphetamine-type appetite suppressants decrease cocaine and methamphetamine self-administration in various animal species [49-53]. Thus, we believed that clinically available appetite suppressants, such as phentermine, diethylpropion, and phendimetrazine, might be logical candi-

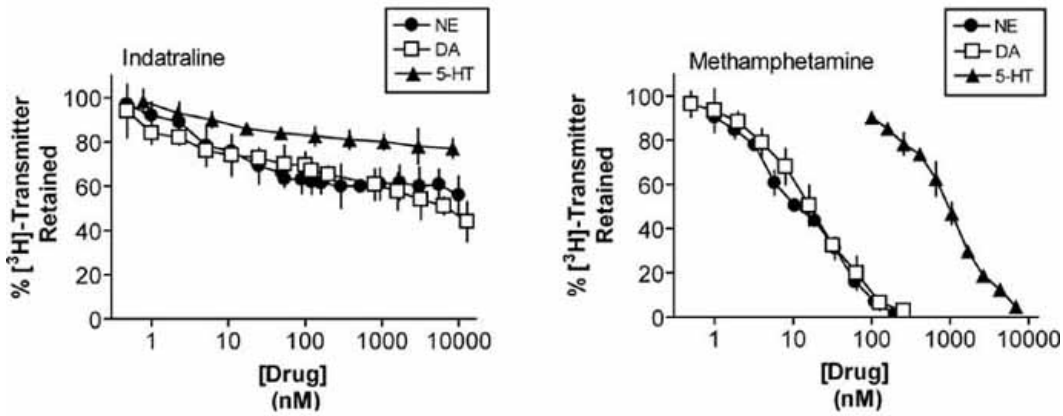


Fig. (2). Effects of the reuptake blocker indatraline (left panel) and the transporter substrate methamphetamine (right panel) on the release of [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]5-HT in synaptosomes. Each point is the mean±SD (n=3). Data are from [44].

Table 2. Pharmacological Profile of Selected Agents in the Dopamine, Norepinephrine and 5-HT Release and Uptake Inhibition Assays

Test Drug	Release NET[a] EC_{50} (nM ± SD)	NE Uptake K_i (nM ± SD)	Release DAT[a] EC_{50} (nM ± SD)	DA Uptake K_i (nM ± SD)	Release SERT EC_{50} (nM ± SD)	5-HT Uptake K_i (nM ± SD)
Appetite Suppressants and their Metabolites						
Phentermine	39.4 ± 6.6		262 ± 21		3511 ± 253	
(+)-Amphetamine	7.07 ± 0.95		24.8 ± 3.5		1765 ± 94	
(-)-Ephedrine	43.1±4.0		236±9		>10,000	>50,000
(+)-Ephedrine	218±14		2104±68		inactive	
Diethylpropion	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000
N-Ethylaminopropiophenone	99.3 ± 6.6			1014 ± 80	2118 ± 98	
N,N-Diethylnorpseudoephedrine	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000
Phendimetrazine	8300 ± 445	>10,000	19,000±537	>10,000	>100,000	>100,000
(±)-Phenmetrazine	50.4±5.4		131±11		7765±610	
Pseudophenmetrazine	514 ± 52			2630 ± 198	>10,000	>10,000
(-)-Pseudophenmetrazine	2511 ± 561			2691 ± 176	>10,000	>10,000
(+)-Pseudophenmetrazine	349 ± 28		1457 ± 138		>10,000	>10,000
Appetite suppressants removed from clinical use						
(+)-Fenfluramine	302 ± 20			22000±1100	51.7 ± 6.1	
(-)-Fenfluramine		7187±559	>10,000	>20,000	147±19	
(±)-Fenfluramine	739 ± 57			23700±1300	79.3 ± 11.5	
(±)-Norfenfluramine	168±17		1925±295		104±5	
(+)-Norfenfluramine	72.7±5.4		924±112		59.3±2.4	
(-)-Norfenfluramine	474±40			19194±1048	287±14	
Aminorex	26.4 ± 2.8		49.4± 7.5		193 ± 23	
Chlorphentermine		451 ± 66	2650 ± 273		30.9 ± 5.4	
Abused Stimulants						
(+)-Methamphetamine	12.3 ± 0.7		24.5 ± 2.1		736 ± 45	
(-)-Methamphetamine	28.5 ± 2.5		416 ± 20		4640 ± 243	
(±)-MDMA	110±10		278±7		72±3	
(+)-MDMA	136±9		142±4		74±3	
(-)-MDMA	560±4		3700±100		340±20	
(±)-MDA	108±7		190±6		160±7	
(+)-MDA	50±5		98±4		100±4	
(-)-MDA	290±10		900±30		310±10	
1-Benzylpiperazine (BZP)	62±6.5		175±13		6050±835	

(Table 2) Contd….

Test Drug	Release NET[a] EC_{50} (nM ± SD)	NE Uptake K_i (nM ± SD)	Release DAT[a] EC_{50} (nM ± SD)	DA Uptake K_i (nM ± SD)	Release SERT EC_{50} (nM ± SD)	5-HT Uptake K_i (nM ± SD)
Endogenous Substrates						
Tyramine	40.6 ± 3.5		119 ± 11		2775 ± 234	
Norepinephrine	164 ± 13		869 ± 51		>10,000	>50,000
Dopamine	66.2 ± 5.4		86.9 ± 9.7			6489 ± 200
Serotonin	>10000	3013 ± 266	1960±147[b]		44.4 ± 5.3	
Transporter Inhibitors						
GBR12935		277 ± 23		4.90±0.30		289±29
GBR12909		79.2 ± 4.9		4.3±0.3		73.2 ± 1.5[1]
Cocaine		779 ± 30		478 ± 25		304 ± 10[2]
Mazindol		2.88 ± 0.17		25.9 ± 0.56		272 ± 11
Methylphenidate[b]		118±12		90.2±7.9		inactive
Desipramine		8.32 ± 1.19		5946 ± 193		350 ± 13
Fluoxetine		688 ± 39		>5,000		9.58 ± 0.88
Citalopram		4332 ± 295		20485 ± 923		2.40 ± 0.09
RTI-55		5.89 ± 0.53		0.83±0.09		1.00 ± 0.03
Indatraline		12.6 ± 0.5		1.90 ± 0.05		3.10 ± 0.09
Miscellaneous Agents						
Nantenine[b]	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
Tramadol[b]	>10,000	2770±250	>10,000	>10,000	>10,000	1820±100
JDTic[b]	>10,000	1756±100[c]	>10,000	3620±230	>10,000	

From: [21, 40, 41, 43, 74, 86, 93, 94]. Each value is the mean±SD of three experiments. For substrates, we report only the release EC_{50} values. For uptake inhibitors, we report the Ki for inhibition of [^{3}H]neurotransmitter uptake.

[a]Some studies used [^{3}H]MPP^+, with appropriate blocking agents, to measure release via DAT and NET.

[b]Unpublished data.

[c]The K_i of JDTic at NET is 6.8 ± 0.9 nM (n=3) using [^{125}I]RTI-55 and the cloned hNET, and is 126 ± 23 nM (n=2) at the rat brain NET labeled with [^{3}H]nisoxetine.

dates for the pharmacotherapy of stimulant dependence [54]. We have performed experiments to examine the molecular mechanisms associated with anorectic drugs.

Phentermine is one of the more widely prescribed appetite suppressant medications. *In vitro* studies (Table **2**) reveal that phentermine is a substrate at NET, DAT and SERT, with its most potent action being NE release (EC_{50} = 39 nM). While the administration of phentermine to rats evokes DA release in the brain [55], the *in vitro* data suggest that phentermine might increase NE release at much lower doses than those required to release DA. The same holds true for (±)-ephedrine, which is at least 10-times more potent at releasing NE than DA. (+)-Amphetamine, in contrast, is only 3-times more potent at NE release than DA release. Unfortunately, to our knowledge, a dose-response comparison of the NE- and DA-releasing effects of these stimulants has not been directly determined using *in vivo* microdialysis methods in rats.

To help clarify this issue, a recent study was conducted in baboons to compare the effects of phentermine, (±)-ephedrine and (+)-amphetamine *in vivo* [56]. Following administration of high (1.5 mg/kg) iv doses of the drugs to anesthetized baboons, both dopaminergic (central DA release and plasma prolactin) and noradrenergic (plasma NE) endpoints were measured. Central DA release was determined via positron emission tomography using the method of [^{11}C]raclopride displacement. As shown in Figs. **5** and **6**, only (+)-amphetamine decreases plasma prolactin and

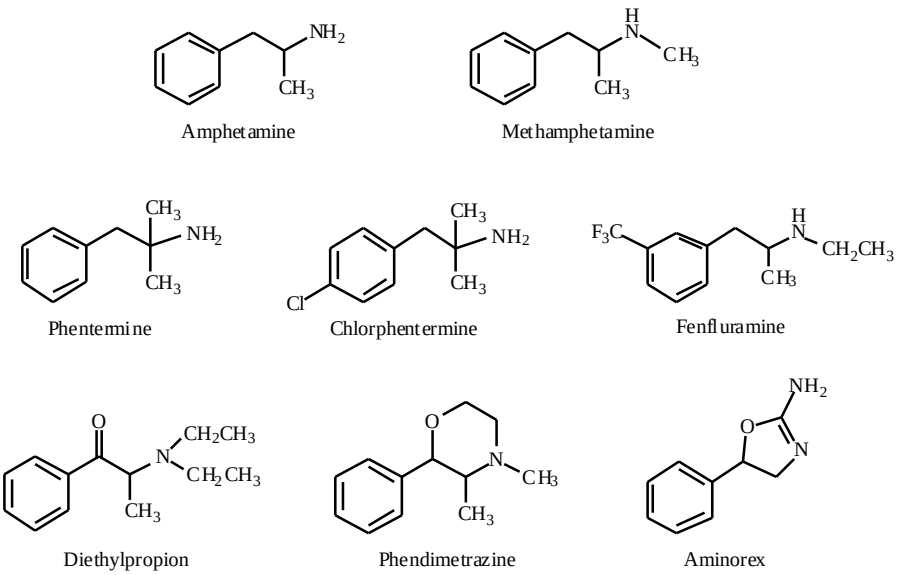


Fig. (4). Structure of selected anorectic agents. From [25].

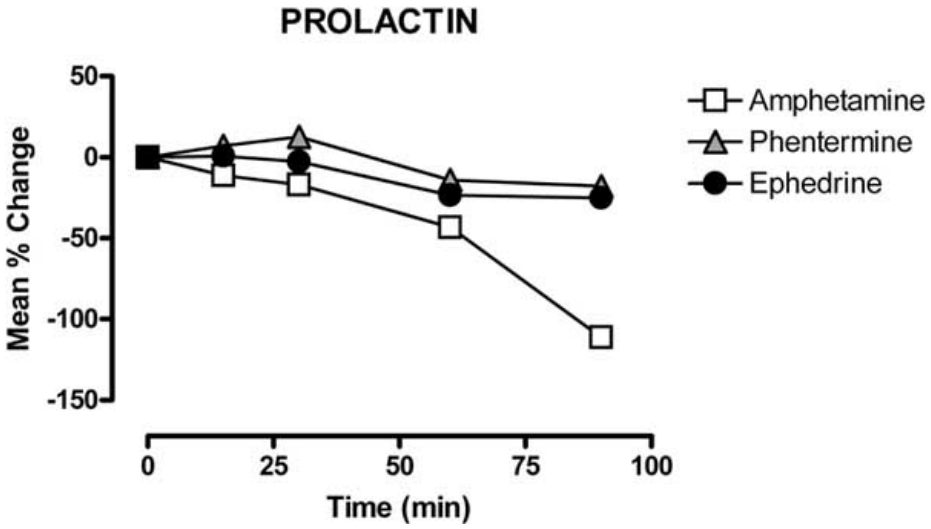


Fig. (5). Mean plasma levels of prolactin in two adult male Papio anubis baboons after the IV bolus administration of amphetamine, (±)-ephedrine, or phentermine. Mean % change = ([prolactin at indicated time] - [prolactin at time 0])*100/[prolactin at time 0]. From [56].

increases central DA release as indicated by reductions in the binding potential of [^{11}C]raclopride. In contrast, all three stimulants increase plasma NE and DA (Fig. **7**). Given that plasma DA levels are about 10-fold less than plasma NE levels, and DA is a precursor in NE biosynthesis, we believe that these stimulants release both NE and DA from peripheral noradrenergic nerves. The plasma levels of the various drugs tested in baboons were at the high end of typical therapeutic plasma levels observed in human subjects (1-3 μM) [57, 58]. These data suggest, therefore, that typical clinical doses of phentermine and (±)-ephedrine may not release central DA in humans. Both phentermine [59] and (±)-ephedrine [60, 61] produce amphetamine-like subjective effects in humans, and the EC_{50} values of stimulants to release NE, but not DA, correlate with the oral doses that produce amphetamine-type subjective effects (Fig. **8**) [40]. Viewed collectively these data support the hypothesis that stimulant-induced increases in NE contribute to the acute subjective effects of amphetamine-like agents. More studies are needed to determine the role of NE in mediating effects of psychomotor stimulants.

Diethylpropion and phendimetrazine are clinically available anorectic agents that display minimal interactions with monoamine transporters *in vitro* (Table **2**). On the other hand, these medications are known to be psychomotor stimulants when administered *in vivo* as indicated by their shared properties with illicit drugs like cocaine. For example, diethylpropion and phendimetrazine are self-administered by animals [45, 62], and both drugs exhibit discriminative stimulus properties that generalize to cocaine [47, 48]. One hypothesis consistent with the available data is that diethylpropion and phendimetrazine are "prodrugs" which are converted to bioactive metabolites upon systemic administration. In the case of diethylpropion, the *N*-deethyla-

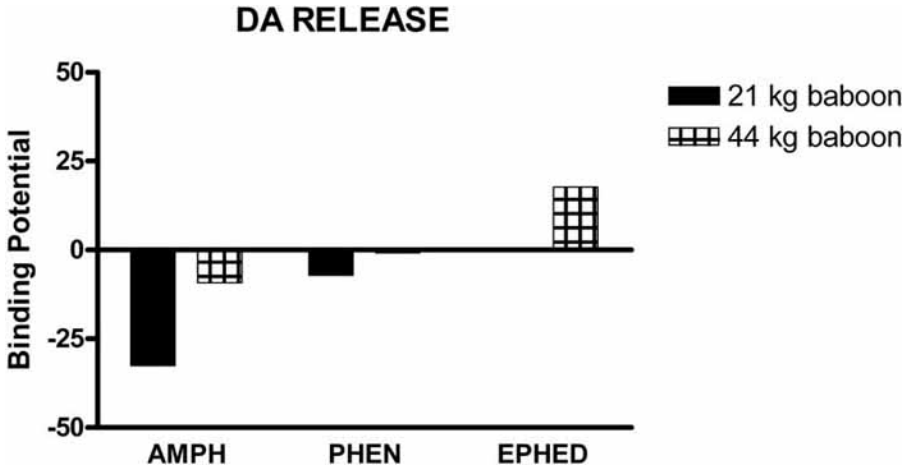


Fig. (6). Dopamine (DA) release as percent change in DA binding potential in two adult male *Papio anubis* baboons following the intravenous bolus injection of 1.5 mg/kg (+)-amphetamine, (±)-ephedrine, or phentermine. From [56].

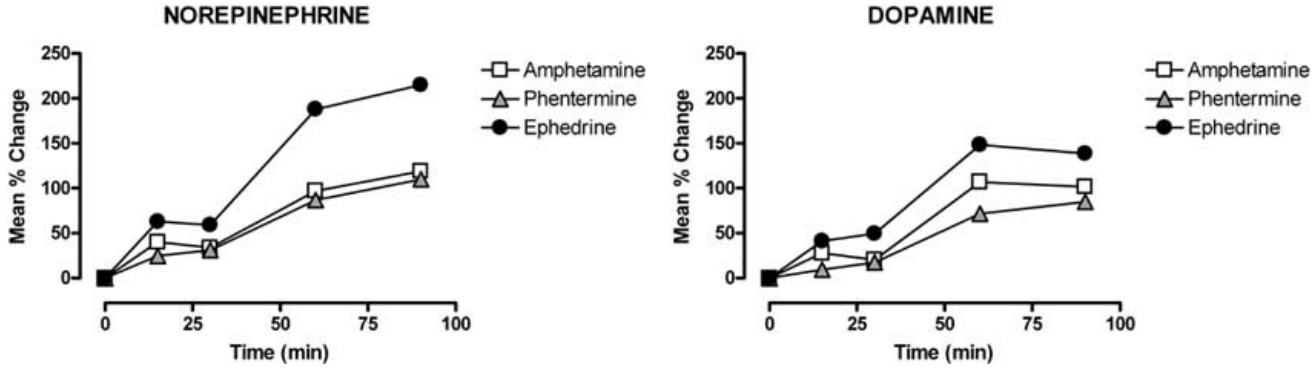


Fig. (7). Mean plasma levels of NE and DA in two adult male *Papio anubis* baboons after the intravenous bolus administration of amphetamine, (±)-ephedrine, or phentermine. Mean % change = ([amine at indicated time] - [amine at time 0])*100/[amine at time 0]. From [56].

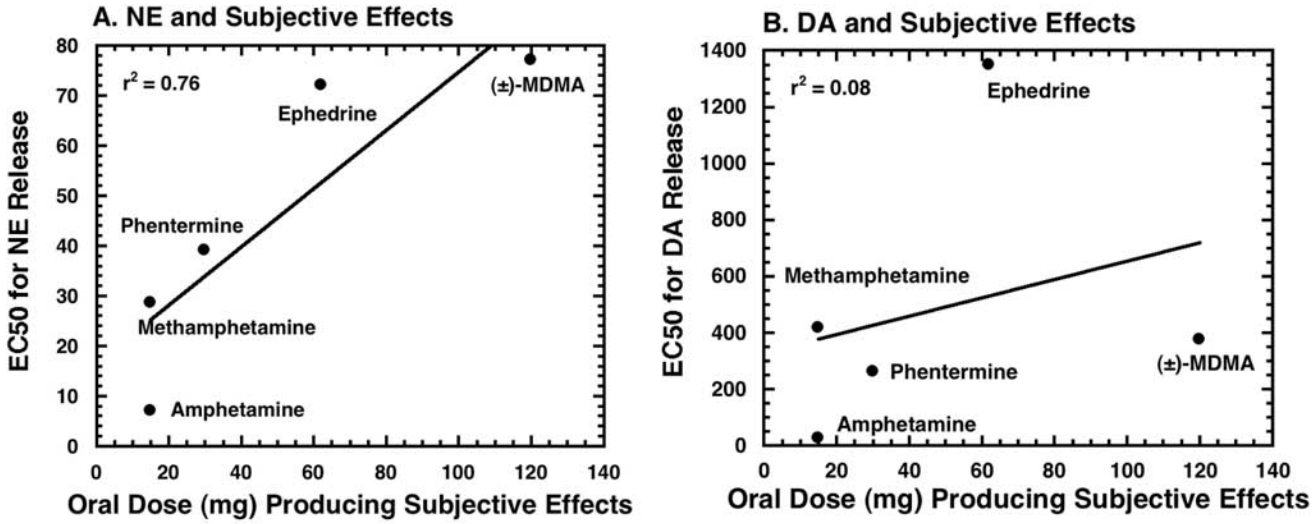


Fig. (8). Correlation of oral doses of stimulants which produce amphetamine-like subjective effects with their potency in releasing [^{3}H]NE (Panel A) and [^{3}H]DA (Panel B). From [40].

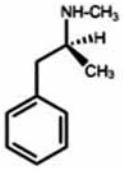
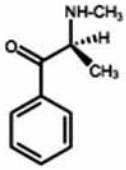
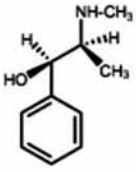
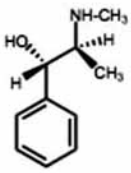
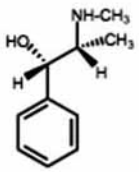
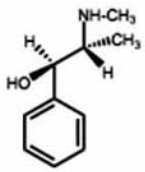
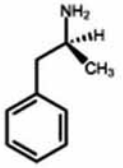
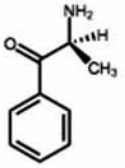
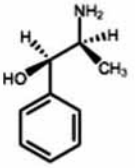
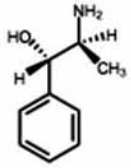
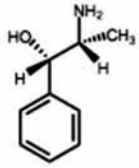
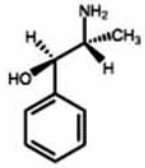
ted metabolite, *N*-ethylaminopropiophenone, appears to be the bioactive metabolite since this compound potently releases NE (EC_{50} = 99.3 nM) with less potent effects on 5-HT release (EC_{50} = 2118 nM). Interestingly, *N*-ethylaminopropiophenone is not a DAT substrate, but instead blocks DA reuptake (EC_{50} = 1014 nM) [63]. In the case of phendimetrazine, the *N*-demethylated metabolite phenmetrazine potently releases NE and DA (Table **2**). Stereochemistry plays an important role in the transporter activity of (±)-phenmetrazine. While the clinically available trans-stereoisomer ((+)-phenmetrazine) is a potent NE and DA releaser, the cis-stereoisomer ((-)-phenmetrazine) is somewhat less active. A more intriguing finding is that enantiomers of (±)-pseudophenmetrazine display different molecular mechanisms at DAT. Specifically, (-)-pseudophenmetrazine is a DA uptake inhibitor whereas (+)-pseudophenmetrazine is a DAT substrate. To our knowledge, this represents the first example of two enantiomers exhibiting differential transporter mechanisms [41].

EPHEDRINE-RELATED COMPOUNDS

Ephedrine is a familiar and widely-used stimulant that was once available in over-the-counter (OTC) preparations. Ephedrine has attracted unfavorable attention because of its use as a synthetic precursor in the clandestine production of methamphetamine [64], and due to its potential for toxicity (including death) [65]. The compound is obtained as an herbal extract derived from plants of the genus *Ephedra* or as a synthetic chemical. All forms of ephedrine have been removed from the OTC market due to the occurrence of cardiovascular side-effects, but the synthetic chemical is still available as a prescription product. Current and past therapeutic applications of ephedrine include treatment of asthma and use as a hypertensive agent, decongestant, central stimulant, and anorectic agent.

Ephedrine has a complex stereochemistry due to the presence of two chiral centers. Specifically, ephedrine-related phenylpropanolamines can exist as four distinct stereoisomers (see Table **3** for chemical structures). [1*R*,2*S*]-(−)-2-(Methylamino)-1-phenylpropan-1-ol is typically identified as (−)-ephedrine, while [1*S*,2*R*]-(+)-2-(methylamino)-1-phenylpropan-1-ol is typically identified as (+)-ephedrine. [1*R*,2*R*]-(−)-2-(Methylamino)-1-phenylpropan-1-ol is (−)-pseudoephedrine, and [1*S*,2*S*]-(+)-2-(methylamino)-1-phenylpropan-1-ol is (+)-pseudoephedrine. *N*-demethylation also results in four optical isomers: [1*R*,2*S*]-(−)-2-(amino)-1-

Table 3. Structure-Activity Profile of Phenylpropanolamines at the Biogenic Amine Transporters EC50 nM (±SD)[a]

Phenylisopropylamines		Phenylpropanonamines	Phenylpropanolamines			
						
S(+)Methamphetamine		**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**	**(+)Pseudonorephedrine**	**(+)Norephedrine**	**(−)Pseudonorephedrine**
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

From [43]. [a]All agents behaved as substrates except for (-)-pseudoephedrine which displayed activity in the DA uptake assay. [b]Data reported previously [40]. 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±S.D. of three experiments.

phenylpropan-1-ol, commonly termed (−)-norephedrine or (−)-phenylpropanolamine, [1*R*,2*S*]-(−)-2-(amino)-1-phenylpropan-1-ol, commonly referred to as (+)-norephedrine or (+)-phenylpropanolamine, (−)-norpseudoephedrine or (−)-cathine, and (+)-norpseudoephedrine or (+)-cathine. Table **3** shows how the structures of the phenylpropanolamines can be related to those of the phenylisopropylamines (i.e. phenylethylamines) methamphetamine and amphetamine. The phenylpropanonamines methcathinone and cathinone differ from methamphetamine and amphetamine 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.

Ephedrine has been extensively studied for decades, but the pharmacological activity of ephedrine-related phenylpropanolamines across a wide array of CNS receptors and transporters was not reported until our study published in 2003 [43]. The most potent action of ephedrine-like phenylpropanolamines is substrate activity at NET. In contrast, methcathinone, cathinone, methamphetamine and amphetamine, release both NE and DA. Importantly, the ephedrine-like phenylpropanolamines have much lower or negligible affinity for adrenergic receptors, indicating that the pharmacological effects of these agents most likely result from the "indirect" NET-mediated release of NE. This latter finding is unexpected, since pharmacology text books teach that the phenylpropanolamines stimulate 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 NE [66].

ADVERSE EFFECTS

The clinical utility of amphetamine-type anorectic agents is limited by a number of adverse side-effects. Cardiovascular complications and high abuse liability are established risks of prescribed NET and DAT substrates (e.g. amphetamine). More recently, adverse effects associated with the use SERT substrates have been widely publicized; these serious side-effects include cardiac valve disease (CVD) and primary pulmonary hypertension (PPH). (±)-Fenfluramine, and its more potent enantiomer (+)-fenfluramine, were once commonly prescribed anorectic agents. These medications were removed from clinical use in 1997 due to the occurrence of CVD in some patients [67]. We reported that fenfluramine, aminorex, and other amphetamine-related drugs known to increase the risk for developing PPH (e.g., chlorphentermine) share the common feature of being SERT substrates [68]. Our findings implicated SERT in the mechanism underlying fenfluramine-induced PPH. It is noteworthy that not all SERT substrates are associated with PPH, and the specific role of SERT proteins in this disease likely involves a complex array of factors that requires further study.

Experimental data from a mouse model of hypoxic pulmonary hypertension have suggested that 5-HT_{2B} receptors are involved in the pathogenesis of PPH [69]. Aminorex is a SERT substrate that caused an epidemic of PPH in the 1960s [70] and case reports implicate the related designer drug, 4-methylaminorex, as a potential cause of the disease [71]. If 5-HT_{2B} receptors are involved in the pathogenesis of aminorex-associated PPH, then we surmised that aminorex should display activity at 5-HT_{2B} sites. As reported in Table **4**, aminorex activates the cloned human 5-HT_{2B} receptor with moderate potency and high efficacy (E_{MAX} = 76%). However, the EC_{50} of aminorex for 5-HT_{2B} receptor activation (870 nM) is 33-times higher than its EC_{50} for NET release (26.4 nM). Moreover, the activity of aminorex at 5-HT_{2B} sites is nearly 50-fold less than the known 5-HT_{2B} agonist (+)-norfenfluramine, the *N*-deethylated metabolite of (+)-fenfluramine. Chlorphentermine is a substituted amphetamine analog that causes PPH in animal models. As shown in Table **4**, this drug has negligible affinity for 5-HT_2 receptor subtypes. Collectively,

Table 4. Functional Activity of Selected Anorectic Agents at the Cloned Human 5-HT_2 Receptors

	5-HT_{2A} (EC_{50} nM) [E_{max}]	**5-HT_{2B} (EC_{50} nM) [E_{max}]**	**5-HT_{2C} (EC_{50} nM) [E_{max}]**	**SERT Release (EC_{50} nM)**	**NET Release (EC_{50} nM)**
5-HT	10.9±0.7 [100±2]	42.6±4.2 [100±4]	0.07±0.01 [100±3]	44.4±5.3[c]	
Aminorex	4365±108 [47±2]	870±27 [76±3]	525±17 [71±2]	193±3[c]	26.4±2.8[c]
Chlorphentermine	inactive	5370±288 [50±5]	6456±237 [48±3]	30.9±5.4[c]	>10000
Phentermine	inactive	inactive	inactive	3511±253[c]	39.4±±6.6[c]
(+)-Norfenfluramine	630±141[a] 88±5	18.4±5.3[a] [73±4]	13±2[a] [100±6]	59.3±2.4[b]	

The data for 5-HT, aminorex and chlorphentermine are previously unpublished. The functional endpoint for these data, provided by the NIMH Psychoactive Drug Screening Program (http://pdsp.cwru.edu/), is Ca^{++} mobilization. [a]The functional endpoint of these data is phosphoinositide hydrolysis [73]. [b]From [42]. [c]From [40]. The values in brackets are the E_{MAX} values, with the effect of 5-HT being 100%. Each value is ±SD.

the data argue against an important role for 5-HT_{2B} receptors in the pathogenesis of anorectic-associated PPH. On the other hand, it seems feasible that a metabolite of aminorex or chlorphentermine may act more potently at 5-HT_{2B} receptors, and this possibility deserves to be examined.

The evidence supporting a role for 5-HT_{2B} receptors in the pathogenesis of fenfluramine-associated CVD is much stronger. As noted above, the association of fenfluramine with an increased prevalence of cardiac valvulopathy led to its withdrawal from the market place. Numerous investigations have demonstrated that the fenfluramine metabolite, norfenfluramine, activates 5-HT_{2B} receptors on heart valves to stimulate mitogensis, and this action may represent the principal mechanism underlying fenfluramine-induced CVD [72-74]. Findings summarized by Setola *et al*. [74] emphasize that valvulopathic agents are "pro-drugs" in many instances, since it is the bioactive metabolites which serve to stimulate 5-HT_{2B} receptor sites. Specific examples of this pro-drug phenomenon are shown in Fig. **9**, where *N*-demethylated metabolites of MDMA and methysergide are potent efficacious 5-HT_{2B} agonists.

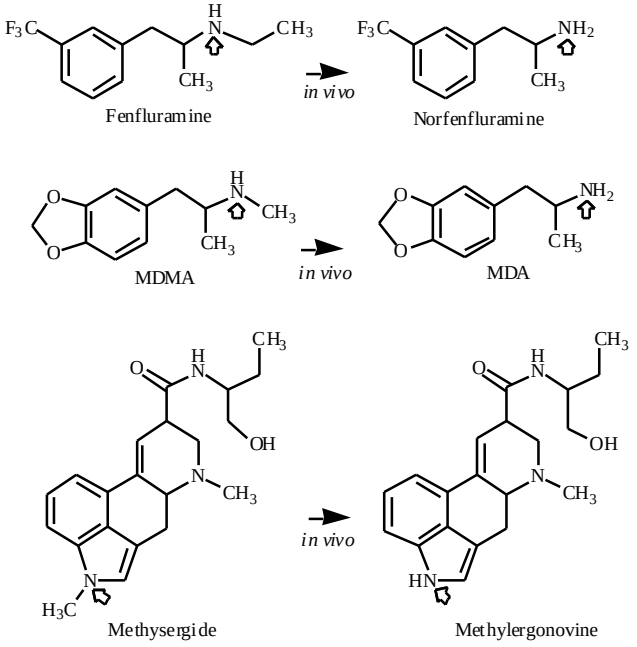


Fig. (9). Metabolism of MDMA, fenfluramine and methysergide results in the formation of norfenfluramine, MDA and methylergonovine. These metabolites are 5-HT_{2B} receptor agonists and are associated with cardiac valve disease. From [74].

As comprehensively reviewed elsewhere [75], certain SERT substrates, such as MDMA and fenfluramine, produce 5-HT neurotoxicity. The term '5-HT neurotoxicity', when used in the present context, refers to the fact that high-dose administration of 5-HT releasers often causes persistent depletion of brain tissue 5-HT and 5-HT transporters. A key observation is that not all SERT substrates deplete 5-HT. For example, repeated administration of the SERT substrate m-chlorophenylpiperazine (mCPP) fails to deplete brain 5-HT, despite producing elevations of extracellular 5-HT comparable to fenfluramine [76]. Other examples of SERT substrates that do not cause 5-HT neurotoxicity include the tetralin and indan analogues of 3,4-(methylenedioxy) amphetamine (MDA) reported by Nichols *et al*. [77] and PAL-287, which is discussed in the next section. Like mCPP, PAL-287 produces elevations in extracellular 5-HT but does not produce long-term 5-HT depletion, even after exposure to very high doses (i.e., 18 mg/kg, i.p. x 3). These data indicate that SERT substrate activity is necessary, but not sufficient to produce long-term depletion of brain 5-HT.

PAL-287 AND RELATED AGENTS

Along with other researchers, we have advocated the use of amphetamine-type monoamine releasers as agonist therapies for cocaine dependence [54, 78]. Similar to cocaine, these compounds target monoamine transporters to elevate synaptic levels of NE, DA and 5-HT [8, 25]. Preclinical studies support the utility of amphetamines as agonist treatments [49, 79]. For example, Negus and Mello [80] demonstrated that slow infusion of the DA releaser (+)-amphetamine decreases cocaine self-administration behavior in monkeys, with minimal effects on food-maintained behavior. Perhaps more importantly, Grabowski *et al*. [81] and Shearer *et al*. [82] showed that (+)-amphetamine is an effective treatment adjunct for reducing illicit cocaine use in cocaine-dependent human patients. Unfortunately, the use of amphetamine as a medication is limited by at least two problems. First, DA releasers possess significant abuse potential due to activation of mesolimbic DA neurons in reward pathways [83]. Second, DA releasers will not correct 5-HT deficits that accompany long-term cocaine abuse [84].

We have suggested the possibility of designing dual DA/5-HT releasers that can overcome both of the aforementioned limitations [85]. For example, a growing body of evidence indicates that increases in 5-HT release can dampen the effects of concurrent DA release, suggesting that mixed DA/5-HT releasers would exhibit less stimulant side-effects. To test whether the balance between DA and 5-HT transmission can predict the occurrence of stimulant-like behaviors, we examined the neurochemical and locomotor effects produced by a series of monoamine releasers that display varying degrees of potency as DAT and SERT substrates. The chemical structures of the drugs tested are shown in Fig. **10**; it should be noted that PAL-313, PAL-314, PAL-303 and PAL-353 are ring-substituted amphetamines whereas PAL-287 is a non-amphetamine compound [21, 86].

Table **5** summarizes *in vitro* potencies of test drugs as releasers of [^{3}H]DA and [^{3}H]5-HT. The DA/5-HT ratio for each drug was calculated by dividing the EC_{50} value for DA release by the corresponding value for 5-HT release. With this method, ratios greater than 1 indicate increasing 5-HT selectivity while ratios less than 1 indicate DA selectivity. All of the drugs are substrate-type releasers that cause efflux of preloaded tritiated transmitter via transporter-mediated mechanisms. The selectivity of drugs for DA and 5-HT transporters differs substantially. (+)-Amphetamine has much higher potency for DA release when compared to 5-HT release (DA/5-HT ratio=0.005). The naphthalene derivative PAL-287 has 500-fold greater potency for 5-HT release when compared to amphetamine, while effects on

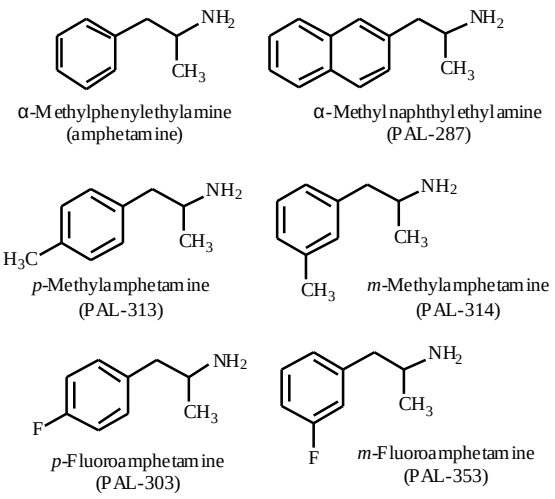


Fig. (10). Chemical structures of (+)-amphetamine, PAL-287, and PAL amphetamines.

DA are comparable (DA/5-HT ratio=3.706). The substituted amphetamines, PAL-313, -314, -303 and -353, display roughly equivalent potency as NE and DA releasers, but these compounds have varying potencies as 5-HT releasers, with PAL-313 exhibiting highest potency and PAL-353 exhibiting lowest potency.

We first compared the *in vivo* neurochemical and behavioral effects of (+)-amphetamine and PAL-287 in rats. Fig. **11** shows that i.v. administration of (+)-amphetamine increases extracellular DA in the prefrontal cortex, with minimal effects on extracellular 5-HT. The microdialysis data are consistent with *in vitro* release data demonstrating (+)-amphetamine is a potent DAT substrate (Table **5**). In

Table 5. Effects of Test Compounds on the Release of [^{3}H]DA and [^{3}H]5-HT From Rat Brain Synaptosomes

Test Drug	[^{3}H]DA release EC_{50} (nM)	[^{3}H]5-HT release EC_{50} (nM)	DA/5-HT ratio
(+)-amphetamine [1]	8.0 ± 0.4	1756 ± 94	0.005
PAL-287 [1]	12.6 ± 0.4	3.4 ± 0.2	3.706
PAL-313 [2]	44.1 ± 2.6	53.4 ± 4.1	0.824
PAL-314 [2]	33.3 ± 1.3	218 ± 22	0.154
PAL-303 [2]	51.5 ± 1.7	939 ± 76	0.055
PAL-353 [2]	24.2 ± 1.1	1937 ± 202	0.012

[1] Data taken from [21]. [2] Data taken from [86]. Data are mean ± SD for N=3 experiments.

amphetamine-treated rats, forward locomotion is markedly stimulated and reaches a level of 6000 cm/20 min after the 1.0 mg/kg dose. Motor activation produced by (+)-amphetamine increases in parallel with elevations in dialysate DA, suggesting a direct relationship between these two endpoints. The data in Fig. **12** illustrate that i.v. administration of PAL-287 increases extracellular levels of DA and 5-HT in prefrontal cortex, with effects on 5-HT being somewhat greater. It is noteworthy that elevations in dialysate DA induced by PAL-287 are similar to those induced by (+)-amphetamine. Despite the large increase in extracellular DA produced by PAL-287, this drug produces very little forward locomotion (Fig. **12**). Specifically, after a 3 mg/kg i.v. dose of PAL-287, dialysate DA levels are increased 8-fold above baseline but ambulation distance is only 1000 cm/20 min. These data provide compelling evidence that stimulation of 5-HT release can antagonize the locomotor stimulation produced by DA release.

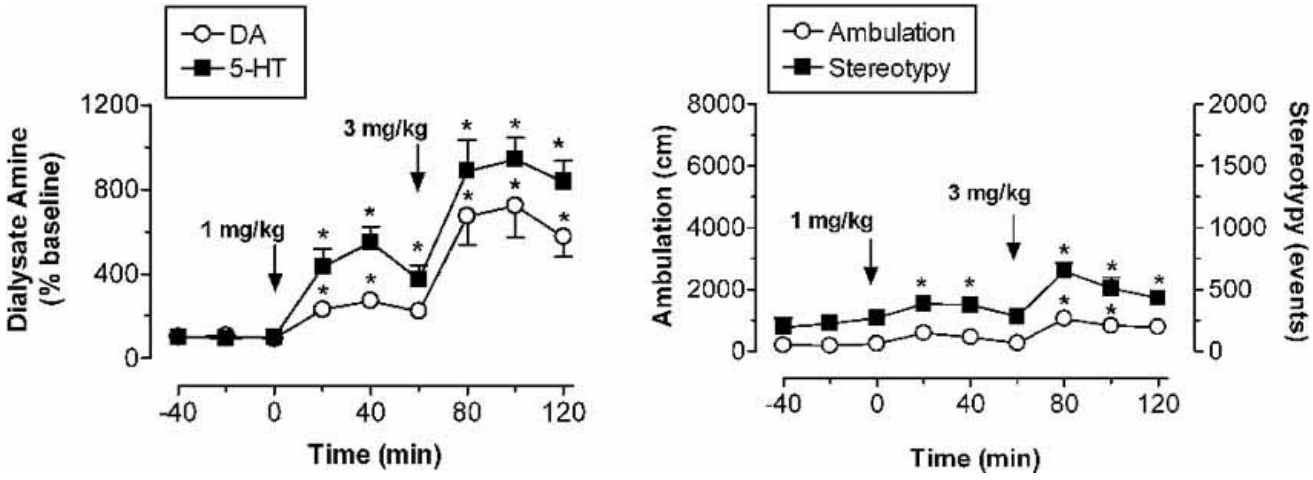


Fig. (11). Left panel. Effects of (+)-amphetamine on extracellular DA and 5-HT in rat prefrontal cortex as determined by *in vivo* microdialysis. Rats received i.v. injection of 0.3 mg/kg (+)-amphetamine at time zero, followed by 1 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as % baseline. Baseline levels of DA and 5-HT were 0.38 ± 0.07 and 0.24 ± 0.06 pg/5 µl. * $P<0.05$ compared to pre-injection control at a given time point, Duncan's post hoc test. Right panel. Effects of (+)-amphetamine on ambulation and stereotypy in rats undergoing microdialysis sampling. Rats received i.v. injections of 0.3 mg/kg (+)-amphetamine at time zero, followed by 1 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as distance traveled in cm (ambulation) and number of repetitive movements (stereotypy). * $P<0.05$ compared to pre-injection control, Duncan's post hoc test. From [21].

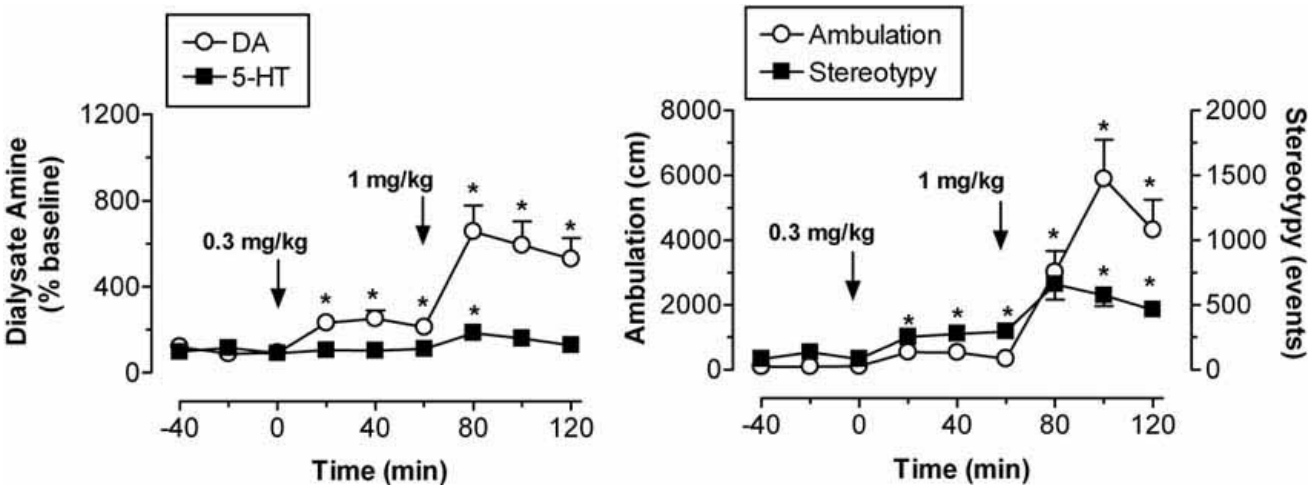


Fig. (12). Left panel. Effects of PAL-287 on extracellular DA and 5-HT in rat prefrontal cortex as determined by *in vivo* microdialysis. Rats received i.v. injection of 1 mg/kg PAL-287 at time zero, followed by 3 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as % baseline. Baseline levels of DA and 5-HT were 0.43 ± 0.07 and 0.27 ± 0.06 pg/5 µl. * P<0.05 compared to pre-injection control at a given time point, Duncan's post hoc test. Right panel. Effects of PAL-287 on ambulation and stereotypy in rats undergoing microdialysis sampling. Rats received i.v. injections of 1 mg/kg PAL-287 at time zero, followed by 3 mg/kg 60 min later. Data are mean ± SEM for 7 rats/group, expressed as distance traveled in cm (ambulation) and number of repetitive movements (stereotypy). * P<0.05 compared to pre-injection control, Duncan's post hoc test. From [21].

The reinforcing effects of PAL-287 have been evaluated in rhesus monkeys. Findings depicted in Fig. **13** demonstrate that PAL-287 is not self-administered by monkeys trained to self-inject cocaine. Similar results were obtained when a series of PAL amphetamines were tested in the monkey self-administration assay. As shown in Fig. **14**, PAL-313 displays the lowest reinforcing efficacy *in vivo*, and this drug is the most potent SERT substrate *in vitro* (Table **5**). The data suggest that serotonergic effects of PAL-287 and PAL-313 blunt the reinforcing effects normally associated with DA release. It should be noted that PAL-287 can readily suppresses on-going cocaine self-administration, even though PAL-287 drug lacks stimulating and reinforcing effects [21]. The findings support the feasibility of developing non-amphetamine releasers with low abuse potential, by designing dual DA and 5-HT releasing activity into a single molecule. Additionally, PAL-287 displays a more desirable side-effects profile when compared to amphetamine-type SERT substrates. PAL-287 has reduced affinity for 5-HT_{2B} receptors and does not persistently deplete brain 5-HT. Taken together, our findings support the possibility of developing dual DA/5-HT releasers that will not produce adverse effects associated with other SERT substrates, such as fenfluramine [22].

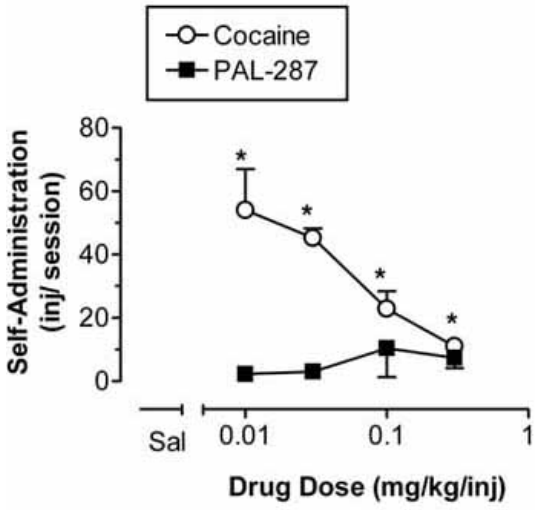


Fig. (13). Self-administration of cocaine and PAL 287 by rhesus monkeys. Drugs were available under a FR 25 schedule of reinforcement for two hours/day. Each point is the mean of two sessions of access to each dose of the drugs. Data are mean ± SEM for N=4 monkeys. Symbols without bars have variability smaller than the points. * P<0.05 compared to saline-injected control, Newman-Keul's post hoc test. From [21].

MISCELLANEOUS DRUGS

We have used our assay procedures to screen a variety of compounds that might possess transporter activity, and previously unpublished data for a few specific agents are mentioned here. The alkaloid nantenine (9,10-methylenedioxy-1,2-dimethoxyaporphine) is a naturally-occurring compound from the fruit of *Nandina domestica* that bears structural similarity to MDMA. Nantenine is reported to antagonize many of the effects of MDMA in mice [87]. Because MDMA is a substrate of the monoamine transporters, nantenine could block the effects of MDMA by acting as a monoamine reuptake inhibitor. As shown in Table **2**, nantenine (supplied by Dr. Fantegrossi) is inactive as a monoamine releaser and as an uptake inhibitor. Tramadol is an analgesic drug sometimes linked to the development of the 5-HT behavioral syndrome [88]. As reported in Table **2**, tramadol inhibits NET and SERT uptake with Ki values in the low micromolar range. Our SERT results are similar to

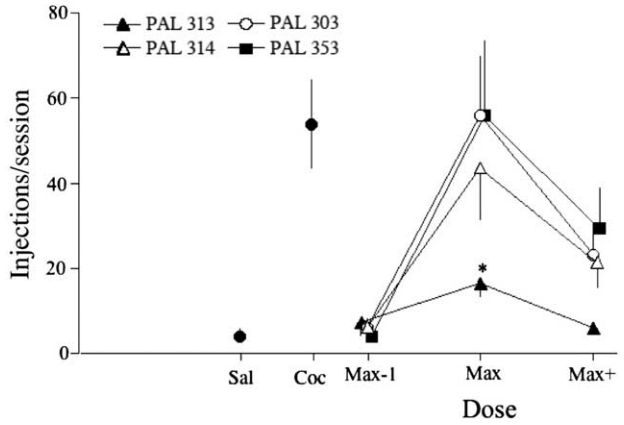


Fig. (14). Self-administration of PAL amphetamines under an FR25 schedule of reinforcement. Drugs were available for self-administration for 2 h/day. Each data point represents the mean injections/session of each dose for four rhesus monkeys, and vertical error bars represent the S.E.M. values. The point above Sal or Coc represents self-administration of saline or the baseline dose of cocaine in test sessions, respectively. Data were normalized as to dose to adjust for individual differences in sensitivity. Max, dose that maintained maximum injections in each animal; Max-1, half-log dose lower than Max; Max+1, half-log dose higher than Max. * P<0.05 compared with PAL 314, PAL 303, or PAL 353, Duncan's post hoc test. From [86].

those reported by Barann *et al*. [89] and indicate that tramadol may inhibit SERT and DAT uptake at therapeutic doses. Finally, JDTic ((3*R*)-7-hydroxy-*N*-((1*S*)-1-([(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl)-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide) is a potent and selective kappa-opioid receptor antagonist [90] that has been reported to have antidepressant-like effects [91]. Such antidepressant activity could be mediated by blockade of monoamine transporters. Interestingly, the K_i of JDTic is 6.8 ± 0.9 nM at cloned human NET labeled with [^{125}I]RTI-55 and 126 ± 23 nM at rat brain NET labeled with [^{3}H]nisoxetine (Table **2**). Although the NET binding assays suggest that JDTic might inhibit NET function at pharmacologically relevant doses, this seems unlikely because JDTic inhibits DAT and NET uptake with Ki values in the low micromolar range, a value 1000-fold higher than its Ki for the kappa receptor (0.02 nM). The difference between the Ki of JDTic in the NET binding assay and the NET uptake inhibition assay probably reflects the different assay conditions used [92].

CONCLUSIONS

Psychomotor stimulants are a fascinating group of compounds that continue to present many challenges to the researcher and clinician. On one hand, psychostimulants can be useful therapeutic agents, while on the other hand, many of these compounds serve as powerful drugs of abuse. We believe that the findings reviewed herein contribute new insights with regard to the mechanism of older amphetamine-type agents. More importantly, these data provide a basis for designing new monoamine releasing agents with diminished adverse effects that could serve as novel treatments for stimulant dependence and other psychiatric disorders.

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