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European Journal of Pharmacology 479 (2003) 23–40
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Monoamine transporters and psychostimulant drugs

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Accepted 21 August 2003

Abstract

Most psychostimulants interact with monoamine transport proteins. This paper reviews work our laboratory has conducted to investigate the interaction of psychostimulants with monoamine transporters in order to advance our understanding of how these drugs affect the brain. We review two topics: (1) characterization of multiple binding sites for cocaine-like drugs and (2) an examination of the mechanisms of action of amphetamine-type anorectic agents. We conclude that the brain contains high abundance nonclassical binding sites for cocaine-like drugs that have micromolar affinity for cocaine and that none of the clinically available amphetamine-type appetite suppressants are equipotent substrates for dopamine transporter (DAT) and serotonin transporter (SERT) proteins. Future medications discovery efforts should focus on identifying new compounds which possess the equipotent substrate activity at DAT and SERT, but which lack the adverse effects of stimulants developed decades ago.

Keywords: Cocaine; Monoamine; Psychostimulant; Amphetamine; Dopamine; 5-HT (5-hydroxytryptamine, serotonin); DAT (dopamine transporter); SERT (serotonin transporter)

1. Introduction

The term ‘‘psychostimulant’’ generally refers to drugs that produce a spectrum of effects in humans that includes increased energy, cardiovascular stimulation, elevated mood, and a decreased need for sleep. At higher doses, or after longer periods of use, psychostimulants can produce a range of disordered thought processes, including severe psychotic episodes. In animals, psychostimulants increase locomotor activity and are readily self-administered due to their powerful reinforcing properties. Psychostimulants are often described as ‘‘amphetamine like’’, since amphetamine is the prototypical stimulant agent. Table 1 lists a number drugs that are classified as psychostimulants. It is noteworthy that many of these drugs are useful medications with long histories of efficacy and safety, whereas others are highly addictive substances that are associated with considerable morbidity and mortality. In some cases, as with amphetamine itself, the same drug can be a therapeutic entity, or an abused substance, depending upon the context in which the drug is administered.

Most psychostimulants are known to interact with monoamine neurons in the central nervous system (CNS). Neurons that synthesize, store, and release monoamine transmitters [norepinephrine, dopamine, and serotonin (5-HT)] are widely distributed in the mammalian CNS. These neurons possess specialized plasma membrane proteins that function to transport previously released transmitter molecules from the extracellular space back into the cytoplasm (Amara and Kuhar, 1993; Masson et al., 1999). Substantial evidence has shown that there are distinct transporter proteins expressed by norepinephrine neurons (i.e., norepinephrine transporters, NETs), dopamine neurons (i.e., dopamine transporters, DATs), and 5-hydroxytryptamine (5-HT) neurons (i.e., 5-HT transporters, SERTs). These proteins belong to a superfamily of Na^+/Cl^--dependent transporters that share genetic, structural, and functional homologies (Blakely et al., 1994; Uhl and Johnson, 1994). Under normal circumstances, the transporter-mediated uptake of monoamine transmitters is the principal mechanism for inactivation of monoaminergic signaling in the brain. Accordingly, a variety of therapeutic and abused drugs interact with monoamine transporter sites (Amara and Sonders, 1998).

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doi:10.1016/j.ejphar.2003.08.054

Table 1
Representative examples of psychostimulants

Therapeutic drugs	Indication
Methylphenidate	Attention deficit disorder
Amphetamine	Attention deficit disorder/narcolepsy
Phentermine	Anorectic
Diethypropion	Anorectic
Phendimetrazine	Anorectic
Benzphetamine	Anorectic
Abused drugs	
Cocaine	
Methamphetamine	
3,4-Methylenedioxymethamphetamine	

Some examples of psychostimulants are provided.

In general, drugs that target transporter proteins can be divided into two classes based on their precise mechanism of action: reuptake inhibitors and substrate-type releasers. Reuptake inhibitors bind to transporter proteins, but are not themselves transported. These drugs elevate extracellular transmitter concentrations by blocking transporter-mediated recapture of transmitter molecules from the synapse. Substrate-type releasers bind to transporter proteins, and these drugs are subsequently transported into the cytoplasm of nerve terminals. Releasers elevate extracellular transmitter concentrations by a two-pronged mechanism: (1) they promote efflux of transmitter by a process of transporter-mediated exchange and (2) they increase cytoplasmic levels of transmitter by disrupting storage of transmitters in vesicles (Rudnick and Clark, 1993; Rudnick, 1997). This latter action increases the pool of neurotransmitter available for release by transporter-mediated exchange. Because substrate-type-releasing agents must be transported into nerve terminals to promote transmitter release, reuptake inhibitors can block the effects of releasers.

For more than a decade, we have carried out experiments in our laboratory to investigate the interaction of psychostimulants with monoamine transporters in order to advance our understanding of how these drugs affect the brain. It is hoped that the knowledge gained from these studies will aid in the development of pharmacotherapies for treating stimulant dependence and other psychiatric disorders. Aspects of this work have recently been reviewed (Prisinzano et al., 2003; Rothman and Baumann, 2002a,b). In the present paper, we will review two specific topics: (1) the characterization of multiple binding sites for cocaine-like drugs and (2) the examination of the mechanisms of action of amphetamine-type anorectic agents.

2. Multiple binding sites for cocaine-like drugs in the CNS

It is well accepted that cocaine is a monoamine reuptake blocker that binds with comparable affinity to NETs, DATs, and SERTs in nervous tissue. Interestingly, most antidepressant medications are also reuptake blockers that bind to one or multiple monoamine transporters. Although not a major focus of recent cocaine research, an unresolved question is why cocaine is not an effective antidepressant (Post, 1975). In fact, the seminal work of Post established that daily administration of cocaine induces psychotic and depression-like syndromes (Post, 1975). As is known to psychiatrists, paranoid ideation and psychosis commonly occur with acute cocaine intoxication, whereas severe depression and suicidality often accompany cocaine withdrawal (Gawin and Kleber, 1986; Latkin and Mandell, 1993; Marzuk et al., 1992).

One hypothesis to explain the aforementioned alterations in mood and thought processes produced by cocaine is the marked stimulation of dopamine transmission afforded by this drug. Similar mood-altering and psychotogenic effects of amphetamine, and the effective treatment of amphetamine psychosis with dopamine receptor antagonists, support this idea. On the other hand, many observations are difficult to reconcile with this hypothesis. For example, a variety of medications that stimulate dopamine transmission, when administered at their usual therapeutic doses, do not induce psychosis or serious depression in most patients. These medications include: nonselective monoamine oxidase inhibitors, selective monoamine oxidase B inhibitors (i.e., selegiline), dopamine uptake blockers like bupropion and nomifensine, and direct dopamine receptor agonists such as pergolide and bromocryptine. Similarly, psychotropic agents that increase synaptic 5-HT, such as selective serotonin reuptake inhibitors (i.e., fluoxetine, sertraline, paroxetine) do not induce psychosis or serious depression in most patients. Antidepressants that display selectivity for NETs in vitro increase extracellular dopamine levels in the frontal cortex, ventral tegmentum and nucleus accumbens in vivo (Chen and Reith, 1994; Li et al., 1996; Rothman et al., 2003). Thus, a broad range of clinically available medications that increase synaptic levels of dopamine, or other monoamines, do not produce the psychotic or depressive syndromes associated with cocaine abuse.

Viewed collectively, these considerations led us to hypothesize the existence of novel high-affinity cocaine binding sites, other than the classic monoamine transporter binding sites, which might contribute to the addictive and psychiatric effects of cocaine. We have used several ligands as probes to identify the postulated novel binding sites (Akunne et al., 1991, 1992, 1994; Dersch et al., 1994). The high-affinity cocaine analog [^{125}I]RTI-55 (3β-(4-^{125}iodophenyl)-tropane-2-carboxylic acid methyl ester) has proven especially useful as a probe for identifying these nonclassical binding sites. Several studies have established that RTI-55 has very high affinity for DATs and SERTs (Boja et al., 1992; Carroll et al., 1992; Rothman et al., 1994a; Staley et al., 1994). Additionally, [^{125}I]RTI-55 displays high specific activity (2200 Ci/mmol) and very low levels of nonspecific binding, making this agent a superb radioligand for characterizing nonclassical binding sites.

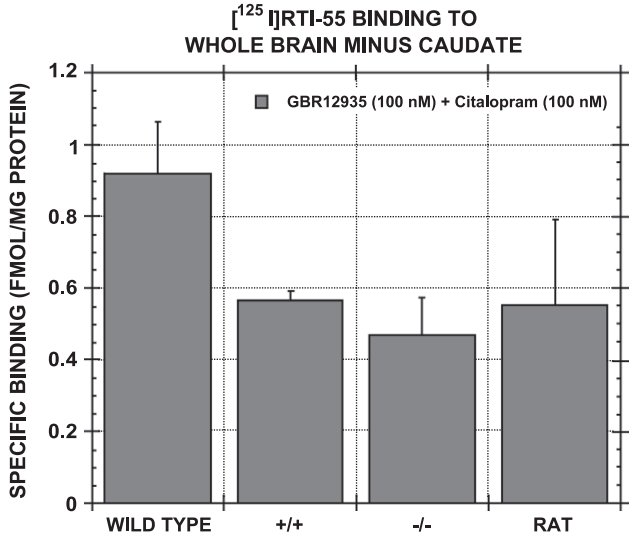

Fig. 1. [^{125}I]RTI-55 binding to whole brain minus caudate. Each value is the mean ± S.E.M. (n = 3). Membranes were prepared from whole brain minus caudate of wild type mice, DAT +/+ and DAT −/− mice, and rat. [^{125}I]RTI-55 binding was determined in the presence of 100 nM GBR12935 and 100 nM citalopram to block DAT and SERT binding, respectively. Data taken from Rothman et al. (2002b).

Our previous work examined [^{125}I]RTI-55 binding to both DAT and SERT proteins in several tissues. In rat caudate membranes (Rothman et al., 1994a), the use of "blocking" ligands permitted selective labeling of DATs and SERTs. Thus, 50 nM paroxetine was used to block [^{125}I]RTI-55 binding to SERTs (DAT assay conditions), whereas 100 nM GBR12935 was used to block [^{125}I]RTI-55 binding to DATS (SERT assay conditions). Under DAT conditions, we observed a single [^{125}I]RTI-55 binding site in rat caudate membranes and in COS cells transiently expressing the cloned rat DAT (Rothman et al., 1994a). However, in membranes prepared from whole rat brain minus the caudate nucleus, we observed a second binding site (Rothman et al., 1995). We initially termed this site DAT_{site2} to indicate a second [^{125}I]RTI-55 binding site measured under DAT assay conditions. It is important to note that this terminology does not necessarily imply a second binding site on the DAT or the presence of an additional DAT protein. Subsequent experiments showed that when DAT_{site2} is compared to the classic DAT binding site in rat brain, DAT_{site2} has a different anatomical distribution, is differentially affected by neurochemical lesions, and has a different ligand-selectivity profile (Rothman et al., 1995). More recent studies using DAT knock out mice demonstrate that what we originally called DAT_{site2} is actually a mixture of two sites: the NET and an unknown site, now termed site 'X' (Rothman et al., 2002b).

Using SERT assay conditions, it is also possible to resolve multiple binding sites for [^{125}I]RTI-55 in membranes prepared from brain tissue (Rothman et al., 1994a; Silverthorn et al., 1995). The most interesting binding site to emerge from the analysis of SERT binding is known as $SERT_{site2}$ (Rothman et al., 1998b). A more detailed discussion regarding the characterization of site 'X' and $SERT_{site2}$ will be considered below.

3. Evidence for site 'X'

As mentioned previously, we first noted the occurrence of a second [^{125}I]RTI-55 binding site in whole rat brain minus caudate membranes under DAT binding conditions (SERT binding blocked with 50 nM paroxetine) (Rothman et al., 1995). To test whether this novel site was related to the classic DAT site, radioligand binding studies were carried out in DAT knockout mice (Rothman et al., 2002b). We prepared membranes from whole brain minus caudate using tissue from wild-type mice, DAT knock out mice (−/−) with their appropriate controls (+/+), and rat brain. Control binding in the absence of blockers was about 20 fmol/mg protein. As reported in Fig. 1, under conditions where DAT and SERT binding is blocked (i.e., in the presence of 100 nM GBR12935 and 100 nM citalopram), there is still measurable [^{125}I]RTI-55 binding in brain membrane preparations. Even though [^{125}I]RTI-55 binding is reduced by 90% in this blocked condition, the residual binding is readily measured because of the low nonspecific binding obtained with [^{125}I]RTI-55.

Binding surface analysis of [^{125}I]RTI-55 binding to whole brain minus caudate membranes prepared from −/− mice under DAT binding conditions (SERT blocked with 100 nM citalopram) demonstrated two binding sites (Table 2). A low-abundance binding site (B_{max} = 64 fmol/mg protein) displayed high affinity for desipramine and moderate affinity for RTI-55 (2.75 nM). A high-abundance binding site (B_{max} = 866 fmol/mg protein) had lower affinity for both desipramine and RTI-55 (21.1 nM). Thus, we surmised that the low-abundance site is the classic NET binding site whereas the high-abundance site is a novel binding site we named site 'X'. In order to characterize the ligand selectivity profile for site 'X', we examined [^{125}I]RTI-55 binding in membranes prepared from DAT knockout mouse brain, where citalopram (100 nM) and DMI (500 nM) were used

Table 2
Best-fit parameter estimates of [^{125}I]RTI-55 binding under DAT conditions in whole brain minus caudate membranes of DAT knockout mice

Parameter	Site 1 (NET)	Site 2 (Site "X")
B_{max} (fmol/mg protein)	63.9 ± 34	866 ± 52
RTI-55 (K_d, nM)	2.75 ± 0.83	21.1 ± 2.7
RTI-229 (K_d, nM)	74.6 ± 28	521 ± 71
Desipramine (K_d, nM)	36 ± 16	734 ± 141

[^{125}I]RTI-55 binding was conducted under DAT binding conditions (SERT binding blocked with 100 nM citalopram). The data of two independent experiments, conducted as described in Section 2, were pooled (280 data points) and fit to the one site and two site binding models. The two site model (SS [sum-of-squares] = 437) fit significantly better (F = 21.9, p < 0.001) than a one site model (SS = 578). Each value is ± the S.D. Data taken from Rothman et al. (2002b).

Table 3
Ligand selectivity of site "X" in DAT knockout mice

Drug	K_i (nM ± S.D.)
Mazindol	41,040 ± 4470
Nisoxetine	153,000 ± 34,000
BTCP	1598 ± 160
Cocaine	2651 ± 174
(+)-Cocaine	19,600 ± 2300
GBR12935	774 ± 104
GBR12909	547 ± 28
Clomipramine	924 ± 67
Methylphenidate	>200,000
RTI-221	211 ± 15
RTI-55	61 ± 5
Indatraline	731 ± 211
Procaine	>50,000
Lidocaine	>50,000
(–)-Cocaethylene	5664 ± 591
Bupropion	>10,000
CFT	>10,000
Fluoxetine	>10,000
PCP	>10,000
RTI-114	>10,000
RTI-117	>10,000
RTI-120	>10,000
RTI-113	>10,000

The data of two independent experiments were combined ($n = 20$) and fit to the two parameter logistic equation for the best-fit estimates of the IC_{50} and slope factor (N). Citalopram (100 nM) and DMI (500 nM) were included to block binding to the SERT and NET. Data taken from Rothman et al. (2002b).

to block binding to SERTs and NETs, respectively. The results shown in Table 3 indicate that local anesthetics, like procaine and lidocaine, have very low affinity for site 'X', suggesting the site it is not a Na^+ channel. High-affinity NET ligands such as mazindol and nisoxetine (Rothman et al., 1998b) exhibit very low affinity for site 'X'. A number of potent DAT ligands (GBR12909, GBR12935, RTI-113, RTI-114, RTI-117, RTI-120) (Rothman et al., 1994a) display very low or negligible affinity for site 'X'. Indatraline, a transporter ligand with high affinity for the DAT, NET and SERT (Rothman et al., 2000), also has very low affinity for site 'X'. SERT-selective ligands such as clomipramine and fluoxetine have low affinity for site 'X'. Interestingly, cocaine and cocaethylene exhibit low micromolar K_i values at site 'X'; these concentrations of cocaine and cocaethylene are in the range achieved in the brain following systemic administration of cocaine in rats (Nicolaysen et al., 1988).

The limited availability of DAT knockout mice led us to develop an assay for site 'X' using wild-type mouse brain. These assays used 100 nM citalopram to block SERT binding, 1 μM nisoxetine to block NET binding, and 1 μM RTI-4229-113 to block DAT binding. Assays were incubated for 4 h at 25 °C (steady state) since this enhanced the signal-to-noise ratio. Initial experiments using these assay conditions demonstrated that [^{125}I]RTI-55 labeled a single binding site with a K_d value of 33.2 nM and a B_{max} value of 1530 fmol/mg protein. These K_d and B_{max} values are similar to those reported for site 'X' in the DAT knockout mouse tissue as reported in Table 2. The data summarized in Table 4 demonstrate that citalopram, nisoxetine, and cocaine exhibit affinities for site 'X' in wild-type mouse brain that are similar to those observed in DAT knockout mouse brain. Out of more than 800 compounds screened for activity at site 'X', only about 10 compounds had moderate potency (i.e., <1 μM) for site 'X'. GBR 12935 and its analogs HY-026TS, YZ-589, DL-649.2, DL-725 and DL-838, have high affinity for DAT, moderate affinities for SERT and NET, and low to moderate affinity for site 'X'. RTI-4229-55 and its analogs RTI-4229-31, RTI-4229-111, RTI-4229-112, RTI-4229-153 and RTI-4229-353, have high affinity for monoamine transporters and moderate affinity for site 'X'.

Although site 'X' has relatively low affinity for [^{125}I]RTI-55 (30–50 nM), the B_{max} of this site (600–1500 fmol/mg protein) exceeds that of the DAT, SERT and NET assayed in the same tissues. Structure–activity studies of site 'X' (Tables 3 and 4) clearly demonstrate that ligands with high affinity for monoamine transporters have low affinity for site

Table 4
[^{125}I]RTI-55 binding to site "X" in wild-type mouse brain

Drug	Site "X", K_i (nM)	DAT, K_i (nM)	SERT, K_i (nM)	NET, K_i (nM)
Citalopram	2231	17,783	0.38	>1000
Nisoxetine	35,524	1325	427	2.3
Cocaine	7765	341	129	13,038
GBR analogs				
GBR 12935	3971	3.7	623	2123
HY 026TS	249	4.9	0.56	13
YZ 589	865	88.9	1903	N.D.
DL 649.2	797	3.8	100	N.D.
DL 725	1338	41	174	N.D.
DL 838	681	5	83	N.D.
Tropane analogs				
RTI-4229-55	50	0.76	0.21	0.80
RTI-4229-31	178	1.12[a]	4[b]	22[c]
RTI-4229-111	29	0.79[a]	0.29[b]	11[c]
RTI-4229-112	31	0.82[a]	0.95[b]	22[c]
RTI-4220-153	164	1.06[a]	0.33[b]	80[c]
RTI-4229-353	114	329[a]	0.69[d]	148[e]

Original [^{125}I]RTI-55 binding data can be found in (Lewis et al., 1999; Rothman et al., 1994b; Zhang et al., 2000). K_i values for site "X" were determined using 0.03 nM [^{125}I]RTI-55 and 100 nM citalopram to block SERT binding, 1 μM nisoxetine to block NET binding. Two separate 8-point inhibition curves were combined and the best-fit estimate of the IC_{50} value determined using MLAB-PC. K_i values were calculated as described in Section 2. Data taken from Rothman et al. (2002b).

[a] K_i value for DAT binding used [^{3}H]WIN 35,428 (Blough et al., 1997; Boja et al., 1994; Carroll et al., 1995).

[b] K_i value for SERT binding used [^{3}H]paroxetine (Boja et al., 1994; Carroll et al., 1995).

[c] K_i value for NET binding used [^{3}H]nisoxetine (Blough et al., 1997; Boja et al., 1994; Carroll et al., 1995).

[d] IC_{50} value for SERT binding using [^{3}H]paroxetine (Blough et al., 1997).

[e] IC_{50} value for NET binding using [^{3}H]nisoxetine (Blough et al., 1997).

'X'. The relatively high density of site 'X' in brain membranes coupled with the fact that K_i values of cocaine and cocaethylene for site 'X' are in the concentration range achieved in the brain following cocaine administration (Nicolaysen et al., 1988) suggests that site 'X' could contribute to pharmacological or toxicological effects of cocaine.

4. Evidence for $SERT_{site2}$

A novel binding site associated with the SERT, known as $SERT_{site2}$ (Rothman et al., 1998b), was detected under SERT binding conditions (i.e., DAT binding blocked with 100 nM GBR12935) in membranes prepared from guinea pig, monkey, or human caudate, but not rat caudate. Fig. 2 illustrates the initial observation that led to the identification of $SERT_{site2}$. In contrast to the monotonic inhibition of [^{125}I]RTI-55 binding under SERT conditions observed in rat brain membranes (Silverthorn et al., 1995), paroxetine inhibited [^{125}I]RTI-55 binding to human caudate membranes in a biphasic manner. Similar results were observed with guinea pig and monkey caudate membranes. Table 5 provides qualitative data supporting the existence of two binding sites under SERT binding conditions in human caudate membranes. Some drugs, in particular the 5-HT uptake inhibitors paroxetine, citalopram, and cloimpramine, had inhibition curves characterized by very low slope factors (0.2 to 0.4), a finding highly predictive of multiple binding sites. In contrast, drugs containing tropane ring structures, such as benztropine and cocaine, had slope factors of 1. Using the technique of binding surface analysis (Rothman et al., 1991), we demonstrated that [^{125}I]RTI-55 labeled two binding sites in membranes prepared from human, monkey or guinea pig caudate. Representative

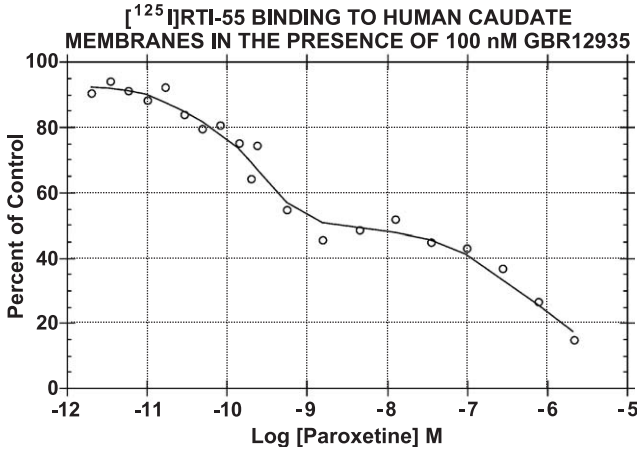

Fig. 2. [^{125}I]RTI-55 binding to human caudate membranes in the presence of 100 nM GBR12935. This graph appears as Fig. 1 in Rothman et al. (1998b). Paroxetine displaces [^{125}I]RTI-55 binding under SERT conditions in a biphasic manner in membranes prepared from human caudate. GBR12935 (100 nM) was used to block [^{125}I]RTI-55 binding to DAT.

Table 5
Qualitative analysis of [^{125}I]RTI-55 binding to human caudate membranes under SERT conditions

Drug	IC_{50} (nM ± S.D.)	Slope factor (N ± S.D.)
Paroxetine	1.4	0.27
Sertraline	56	0.38
Clomipramine	144	0.25
Benztropine	5400	1.02
Nomifensine	869	0.68
Cocaine	4765	1.17
CFT	119	0.74
WIN35,065-2	235	0.86

[^{125}I]RTI-55 (0.01 nM) was displaced by 8 to 10 concentrations of the indicated drugs. GBR12935 (100 nM) was present in each assay tube to block [^{125}I]RTI-55 binding to the dopamine transporter. Each inhibition curve was generated two to three times. The data were pooled and fit to the two parameter logistic equation for the best-fit estimates of the slope factor (N) and IC_{50}. Data are from Rothman et al. (1998b).

results observed in guinea pig caudate membranes are reported in Table 6. Several key findings emerged from this analysis. First, although RTI-55 has about 50-fold lower affinity for $SERT_{site2}$ when compared to SERT (K_d = 0.12 nM), the drug still displays reasonably high affinity for $SERT_{site2}$ (5.73 nM). By way of comparison, the K_d of RTI-55 for DAT is 0.76 nM (Rothman et al., 1994a). Second, the B_{max} of $SERT_{site2}$ (1060 fmol/mg protein) is about 10-fold higher than the B_{max} of the classic SERT, indicating that $SERT_{site2}$ is a high-abundance binding site. Finally, $SERT_{site2}$ is a site easily distinguished from the classic SERT binding site due to its low affinity for paroxetine and ketanserin. Similar results were observed in human and monkey caudate membranes.

Given the high density of DAT binding sites in caudate membranes, it was obviously important to rule out the possibility that $SERT_{site2}$ was merely residual DAT binding (i.e., DAT binding not blocked by 100 nM GBR12935). We tested the hypothesis that $SERT_{site2}$ and DAT are distinct binding sites using several strategies. The data summarized in Fig. 3 present one line of evidence demonstrating that $SERT_{site2}$ and DAT are unrelated binding sites. Using a range of compounds, we compared the K_i values of test agents at $SERT_{site2}$ and DAT. In Fig. 3, the data are

Table 6
[^{125}I]RTI-55 binding to SERT-related binding sites in guinea pig caudate membranes

	Site 1 (SERT)	Site 2 ($SERT_{site2}$)
B_{max} (fmol/mg protein)	100 ± 2.0	1060 ± 44
RTI-55 (K_d, nM)	0.12 ± 0.003	5.73 ± 0.30
Paroxetine (K_i, nM)	0.97 ± 0.05	4208 ± 318
Ketanserin (K_i, nM)	173 ± 3.4	16,986 ± 1079

[^{125}I]RTI-55 binding to guinea pig caudate membranes was carried out in the presence of 100 nM GBR12935 to block binding to the dopamine transporter. Fitting the data (528 data points) to a one-site model resulted in an SS of 2.34E4. Fitting the data to a two-site binding model resulted in an SS of 1.29E3. This was a highly significant decrease in the SS (F = 2433, p = 0). The best-fit estimates of the parameter values (± S.D.) are reported above. Data are from Rothman et al. (1998b).

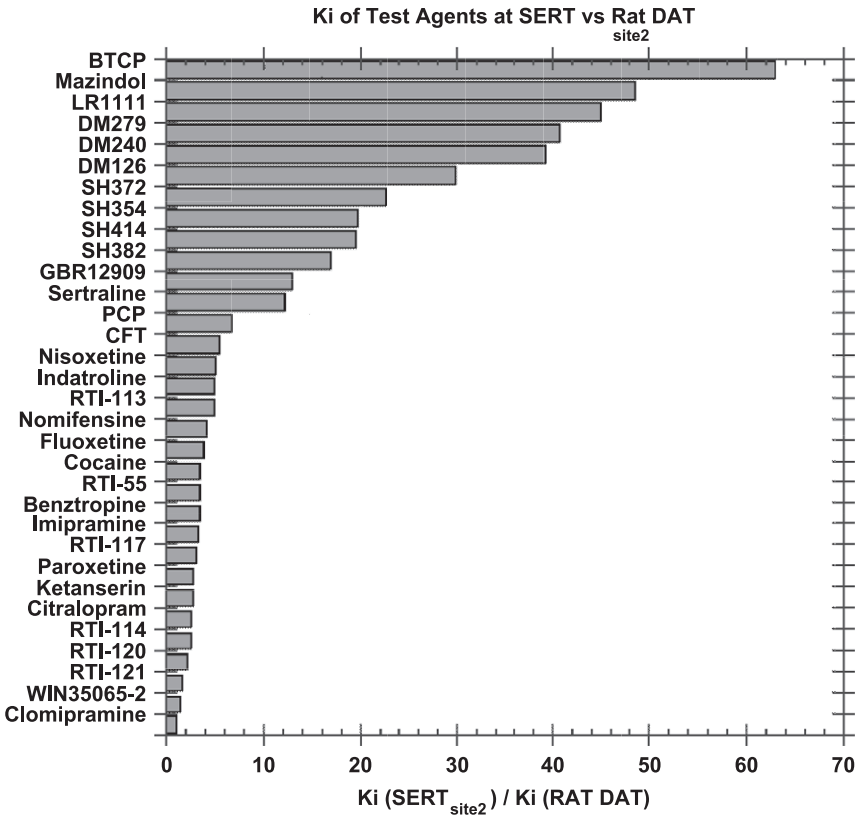

Fig. 3. K_i of test agents at $SERT_{site2}$ and rat DAT. This graph appears as Fig. 2 in Rothman et al. (1998b). The K_i values of various test agents for $SERT_{site2}$ (determined in guinea pig caudate membranes) is compared to the K_i values at the rat DAT. $SERT_{site2}$ binding was determined using [^{125}I]RTI-55 and blockers of SERT (50 nM paroxetine) and DAT (100 nM GBR12935) binding.

expressed as a ratio of K_i ($SERT_{site2}$)/K_i (DAT). If DAT and $SERT_{site2}$ are the same site, then this ratio should be relatively constant across the entire range of compounds tested. The results clearly show that the K_i ($SERT_{site2}$)/K_i (DAT) ratios varied from 1 to over 60. The data summarized in Table 7 show that certain compounds that possess high affinity for DAT have substantially lower affinity for $SERT_{site2}$. For example, the GBR12909 analogs (LR1111, DM126, DM279, DM240) are 30–45 fold less potent at $SERT_{site2}$ when compared to DAT. Similarly, the "SH" BTCP (1-[1-(2-benzo[*b*]thiopheneyl)cyclohexyl]piperidine) analogs are 17–23 fold less potent at $SERT_{site2}$ than DAT.

Having ruled out the proposal that $SERT_{site2}$ is related to residual DAT, we sought to determine the relationship between $SERT_{site2}$ and other known CNS binding sites. We first screened a battery of agents (10 μM) at monkey caudate $SERT_{site2}$. The data depicted in Table 8 demonstrate that high-affinity ligands for σ receptors ((+)-pentazocine), acetylcholine receptors (atropine), α-adrenoreceptors and β-adrenoreceptors (phenoxybenzamine, propranolol), non-competitive NMDA receptors ((+)-MK801), and dopamine receptors (chlorpromazine) have minimal or little activity at $SERT_{site2}$. The findings with chlorpromazine are of interest since this agent is a "dirty" drug, with high affinity for a wide range of CNS receptors, including histamine receptors. Metergoline, a nonselective 5-HT receptor antagonist, has the greatest potency at $SERT_{site2}$. Table 9 shows the data from a comprehensive structure–activity investigation of ligand binding at DAT, SERT, NET and $SERT_{site2}$. Inspec-

Table 7
K_i values of selected agents for the rat caudate DAT and the guinea pig caudate $SERT_{site2}$

Drug	K_i at Rat DAT (nM ± S.D.)	K_i at $SERT_{site2}$ (nM ± S.D.)	K_i at Rat DAT/K_i at $SERT_{site2}$
SH372	18.7 ± 1.3 [n = 1.18]	423 ± 39 [n = 1.31]	22.6
SH382	26.9 ± 1.8 [n = 1.36]	456 ± 19 [n = 1.22]	16.9
SH354	23.9 ± 1.7 [n = 1.26]	470 ± 42 [n = 1.10]	19.6
SH414	25.3 ± 2.4 [n = 1.19]	495 ± 23 [n = 0.88]	19.5
LR1111	9.64 ± 0.29 [n = 1.19]	432 ± 19 [n = 1.08]	44.8
DM126	3.14 ± 0.08 [n = 1.12]	93.8 ± 4.3 [n = 1.15]	29.8
DM279	4.96 ± 0.16 [n = 1.30]	202 ± 9.9 [n = 1.10]	40.7
DM240	1.15 ± 0.03 [n = 1.08]	45.0 ± 3.0 [n = 0.76]	39.1

[^{125}I]RTI-55 (0.011 nM) binding to the rat DAT was determined using rat caudate membranes and 50 nM paroxetine to block binding to the SERT. [^{125}I]RTI-55 (0.011 nM) binding to the $SERT_{site2}$ was determined using 100 nM GBR12935 and 50 nM paroxetine to block binding to the DAT and SERT, respectively. Initial experiments were conducted to determine the concentration ranges of each test agent at both binding sites. Each inhibition curve consisted of eight data points. The data of two experiments were pooled (16 points) and fit to the logistic equation for the best-fit estimates of the IC_{50} and slope factor (N). The K_i values (± S.D.) are reported above. The slope factors did not significantly differ from 1.0. These data are from Rothman et al. (1998b).

Table 8
Interaction of miscellaneous ligands with $SERT_{site2}$

Drug	Percent inhibition at 10 μM
(+)-Pentazocine (high affinity σ receptor ligand)	4.05
Atropine (acetylcholine receptor antagonist)	5.3
Phenoxybenzamine (α-adrenoreceptor antagonist)	55.6
(+)-MK801 (noncompetitive NMDA receptor antagonist)	19.4
(–)-Propranolol (β-adrenoreceptor and 5-HT_1 receptor antagonist)	31.1
Chlorpromazine (dopamine receptor antagonist)	48.9
Metergoline[a] (nonselective 5-HT receptor antagonist)	81.3

[^{125}I]RTI-55 (0.01 nM) binding to monkey caudate $SERT_{site2}$ was determined with blockers of SERT (50 nM paroxetine) and DAT (50 nM RTI-122). Each point is the mean of two experiments that differed by less than 10%. These data are from Rothman et al. (1998b).

[a] $IC_{50} = 1492 \pm 311$ nM.

tion of these data reveals that a diverse range of compounds with established receptor selectivity have very low affinity for $SERT_{site2}$. These compounds include benzodiazepine receptor agonists (alprazolam, clonazepam), monoamine oxidase A and monoamine oxidase B inhibitors (clorgyline, pargyline, phenelzine, tranylcypromine), vesicular monoamine transporter inhibitors (reserpine, ketanserin), various dopamine receptor antagonists (haloperidol, clozapine, pimozide, chlorpromazine), a wide range of antidepressants, and cocaine metabolites. It should be noted that the dopamine receptor antagonists are nonselective agents that display appreciable affinity for adrenergic, serotonergic and histaminergic receptors.

The data reviewed above indicate that of the compounds examined, none has higher affinity for $SERT_{site2}$ than for the DAT. Hoping to identify a potent and selective $SERT_{site2}$ ligand, we utilized a positional-scanning combinatorial hexapeptide library and tested the peptides in binding assays (Rothman et al., 1999b). This effort failed to identify a selective $SERT_{site2}$ agent. However, the data demonstrated a poor correlation between $SERT_{site2}$ binding potency and DAT binding potency, providing further evidence that DAT and $SERT_{site2}$ are different binding sites (Fig. 4).

Taken together, these findings indicate that $SERT_{site2}$ is a unique high-abundance binding site for [^{125}I]RTI-55. Presently, we have not identified any agent that has higher affinity for $SERT_{site2}$ than for the DAT. As a result, while it is possible to construct blocking conditions that permit selective labeling of $SERT_{site2}$ with [^{125}I]RTI-55, it is not possible to generate truly selective labeling conditions for the DAT. A point of interest is that our data predict [^{125}I]RTI-121, which is being used as a selective ligand for the DAT (Boja et al., 1995), will also label $SERT_{site2}$ (Rothman et al., 1999b).

A striking finding with regard to $SERT_{site2}$ is the wide range of representative antidepressant agents and other drugs that exhibit very low affinity for this binding site. The affinity of cocaine for $SERT_{site2}$ (about 1 μM) is similar to the concentration of cocaine achieved in the brain at pharmacological doses (Nicolaysen et al., 1988). In view of the fact that high affinity $SERT_{site2}$ ligands are mostly cocaine analogs, we speculate that actions of cocaine that differ from the classic monoamine uptake inhibitors may be mediated in part via $SERT_{site2}$. Further progress in studying

Table 9
Interaction of antidepressants and other agents with monoamine transporters and $SERT_{site2}$

Drug	K_i (nM) DAT	K_i (nM) NET	K_i (nM) SERT	K_i (nM) $SERT_{site2}$
Alprazolam	>10,000	>10,000	>10,000	>10,000
Amantadine	>10,000	>10,000	>10,000	>10,000
Amfonelic acid	3089	15,294	16,209	55,006
Amitriptyline	5472	27	107	22,412
Amoxapine	5411	24.8	1108	33,717
Bupropion	1534	>10,000	34,707	19,271
Chlorpromazine	2965	10.4	732	12,079
Clomipramine	3398	54	1.1	11,892
Clonazepam	>10,000	>10,000	>10,000	>10,000
Clorgyline	15,106	>10,000	1500	38,429
Clozapine	15,967	653	3279	99,707
Cocaethylene	555	>10,000	3878	4245
Desipramine	5312	0.77	288	>10,000
Doxepin	11,677	40	355	141,509
Ecgonine	>10,000	>10,000	>10,000	>10,000
Ecgonidine	>10,000	>10,000	>10,000	>10,000
Fluoxetine	5437	1235	17.7	24,759
Haloperidol	3248	9303	3449	26,947
Imipramine	10,769	28	37.2	>10,000
Maprotiline	6099	14.2	4198	12,732
Mazindol	37.6	0.6	631	414
Mianserin	6505	124	5092	33,393
Nomifensine	93.1	32	1889	1235
Nortriptyline	2129	4.34	190	7305
Pargyline	>10,000	>10,000	>10,000	>10,000
Paroxetine	876	161	0.033	4654
Phenelzine	>10,000	>10,000	>10,000	>10,000
Pimozide	99	2689	37.9	995
Protriptyline	3030	1.82	155	18,153
Reserpine	4434	>10,000	189,338	6206
Tranylcypromine	30,824	7651	>10,000	>10,000
Trazadone	19,387	32,357	321	35,129
Trimipramine	5783	5708	4234	54,967
Xylamine	68,257	48,154	>10,000	>10,000
Zimelidine	not done	33,593	289	>10,000

The SERT assay used rat caudate membranes, [^{125}I]RTI-55 (0.01 nM) and 100 nM GBR12935 as a blocker. The NET assay used membranes prepared from whole rat brain and 1 nM [^{3}H]nisoxetine. The DAT assay used rat caudate membranes, 0.01 nM [^{125}I]RTI-55 and 50 nM paroxetine. The $SERT_{site2}$ assay used membranes prepared from guinea pig caudate, [^{125}I]RTI-55 (0.01 nM) and GBR12935 (100 nM) and paroxetine (50 nM) as blockers. These data are from Rothman et al. (1998b).

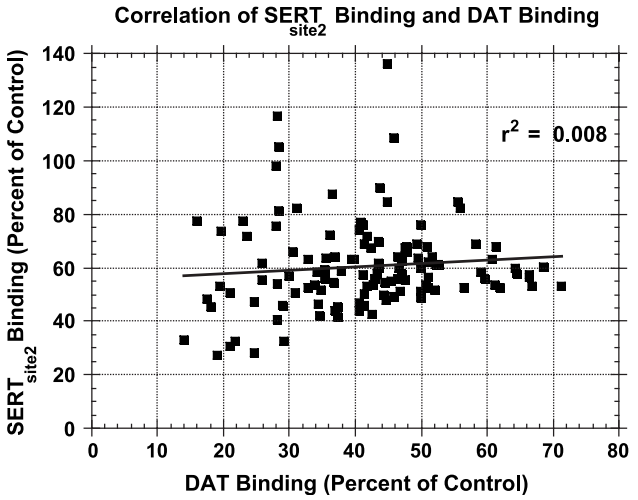

Fig. 4. Correlation of $SERT_{site2}$ binding and DAT binding. The pattern of inhibition produced by the hexapeptide library PS-SCL at DAT and $SERT_{site2}$ were poorly correlated ($r^2 = 0.008$). This is Fig. 2 from Rothman et al. (1999b).

$SERT_{site2}$ will depend on identifying new potent and selective ligands.

5. Summary: multiple binding sites for cocaine-like ligands

The work reviewed above establishes the existence of two nonclassical binding sites for the cocaine analog [^{125}I]RTI-55. Both site 'X' and $SERT_{site2}$ are high-abundance binding sites that display K_i values for cocaine in the low-micromolar range, indicating these sites could be relevant to the pharmacological actions of cocaine. Unfortunately, there are significant impediments to the continued study of these two binding sites. For both site 'X' and $SERT_{site2}$, the lack of selective high-affinity radioligands is a key limiting factor. [^{125}I]RTI-55 has such poor affinity for site 'X' that if not for the very low nonspecific binding of this ligand, we would not be able to detect the site. For both novel-binding sites, the lack of potent and selective ligands precludes the determination of functional correlates for these sites in vivo. Another difficulty with studying $SERT_{site2}$ is that it does not occur in rats, a commonly used laboratory animal. Despite these difficulties, the data obtained to date demonstrate that there remains much to learn about nonclassical binding sites for cocaine-like agents.

6. Mechanism of amphetamine-type anorectics

Cocaine and methamphetamine are generally acknowledged to be among the most addictive substances known (Das, 1993; Gonzalez Castro et al., 2000; Musto, 1992). Long-term stimulant abuse is associated with considerable morbidity and mortality (Anonymous, 1995; Das, 1993). The co-morbidity of drug abuse with psychiatric disorders, as well as the clinical presentation of primary stimulant dependence (Kalechstein et al., 2000; Miller, 1994; Regier et al., 1990), indicates that efforts to develop new and effective treatments for stimulant addictions is an important goal of biomedical research. The association of intravenous drug use with the spread of human immunodeficiency virus (HIV) (Batki, 1990; Halkitis et al., 2001) heightens the importance of this effort to society.

The use of stimulant-like medications to treat stimulant addictions is an approach described as "agonist substitution" therapy. This strategy involves administering medications which are less potent and less addictive than cocaine or methamphetamine, but which nevertheless decrease stimulant abuse because of shared neurochemical properties with the illicit drugs (Gorelick, 1998). Viewed from this perspective, agonist substitution therapy could be described as neurochemical "normalization" therapy—by substituting for the abused drug, the treatment drug "normalizes" dysregulated neurochemistry. Neurochemical normalization therapy has generated effective treatments for nicotine dependence (Henningfield, 1995) and opioid dependence (Kreek, 1996; Ling et al., 1994), and this approach has recently been explored for the treatment of cocaine dependence (Alim et al., 1995; Grabowski et al., 1997, 2001; Kampman et al., 2000; Walsh et al., 2000).

An important first step in the development of successful normalization therapy for psychostimulant addictions is determining the neurobiological consequences of long-term stimulant abuse. Converging lines of evidence indicate that cocaine withdrawal is accompanied by impairments in dopamine and 5-HT function in the brain (Baumann et al., 1995; Baumann and Rothman, 1998b; Cunningham et al., 1992; Kuhar and Pilotte, 1996; Levy et al., 1994; Parsons et al., 1995; Weiss et al., 1992). For example, in vivo microdialysis studies in rats have shown that withdrawal from repeated cocaine injections is accompanied by decreases in basal extracellular levels of both dopamine and 5-HT in the brain (Parsons et al., 1991, 1995; Robertson et al., 1991; Rosetti et al., 1992). Based on these findings, we proposed a dual deficit model of stimulant withdrawal in which drug-induced dopamine and 5-HT dysfunction contributes to withdrawal symptomatology, drug craving, and relapse (Baumann and Rothman, 1998a,b; Baumann et al., 2000; Rothman et al., 1998a). According to the dual deficit model depicted diagrammatically in Fig. 5, decreased synaptic dopamine during stimulant withdrawal underlies anhedonia and psychomotor retardation, whereas decreased synaptic 5-HT gives rise to depressed mood, obsessive thoughts, and lack of impulse control. Consistent with this model, rats receiving repeated injections of abused stimulants exhibit neurobiological changes similar to those observed in human patients with major depression (Baumann and Rothman, 1998a; Levy et al., 1994; Lin et al., 1999; Markou and Koob, 1991). The dual deficit model predicts that pharmacotherapies capable of normalizing the proposed abnormal-

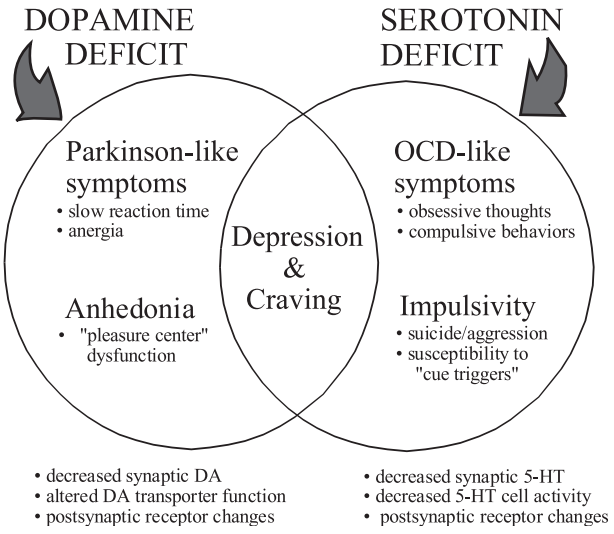

Fig. 5. The dual deficit model of psychostimulant withdrawal. According to the model, withdrawal from chronic stimulant use leads to decreased synaptic availability of dopamine and 5-HT that, in turn, contributes to withdrawal symptoms, drug craving, and relapse. Dopamine dysfunction underlies anhedonia and psychomotor disturbances, whereas 5-HT dysfunction causes depressed mood, obsessive thoughts, and lack of impulse control. Protracted withdrawal phenomena are postulated to contribute significantly to relapse. From Rothman et al. (2002a).

ities in dopamine and 5-HT function should be effective in treating stimulant dependence. Indeed, drugs that release dopamine (phentermine, amphetamine) and 5-HT (fenfluramine) display properties that are consistent with effective treatment of substance use disorders (Halladay et al., 1999; Hitzig, 1993; Rothman et al., 1994c, 1998a; Yu et al., 1997).

Clinically available appetite suppressants, such as phentermine, diethylpropion, and phendimetrazine, are logical candidates for neurochemical normalization therapy of stimulant dependence. As depicted in Fig. 6, many anorectic medications are structurally related to amphetamine (i.e., phenylethylamines). Furthermore, these drugs share behavioral properties with abused stimulants, but are less potent and less addictive (Corwin et al., 1987; Evans and Johanson, 1987; Griffiths et al., 1976; Wood and Emmett Oglesby, 1988). A number of preclinical studies have shown that amphetamine-type appetite suppressants decrease cocaine and methamphetamine self-administration in various animal species (Glatz et al., 2002; Glowa et al., 1997; Munzar et al., 1999; Stafford et al., 2001; Wojnicki et al., 1999). Depending on the specific anorectic drug tested, these agents can increase extracellular levels of dopamine, 5-HT, or both transmitters, in the brain (Balcioglu and Wurtman, 1998; Baumann et al., 2000; Rothman et al., 1999a; Shoaib et al., 1997). According to the dual deficit hypothesis, the ideal normalization pharmacotherapy should elevate extracellular levels of dopamine and 5-HT to a similar extent, and the balance between stimulation of dopamine and 5-HT transmission has important therapeutic implications. For example, anorectic agents like phentermine that stimulate dopamine transmission possess the undesirable qualities of locomotor activation and abuse liability. Drug treatments that stimulate 5-HT transmission, on the other hand, can antagonize phentermine-induced motor activation (Baumann et al., 2000), phentermine-related reward (Rothman et al., 1998a) and phentermine-associated subjective effects (Brauer et al., 1996). Thus, elevations in synaptic 5-HT appear to counteract some undesirable effects mediated by elevations in synaptic dopamine. Consistent with this hypothesis, the phentermine/fenfluramine combination is not self-administered by rats (Glatz et al., 2002).

As noted previously, illicit stimulants like cocaine and methamphetamine interact with DAT, NET, and SERT in nervous tissue (Amara and Sonders, 1998; Rudnick and Clark, 1993). More specifically, cocaine binds to monoamine transporters thereby blocking transmitter reuptake. Methamphetamine acts as a substrate for monoamine trans-

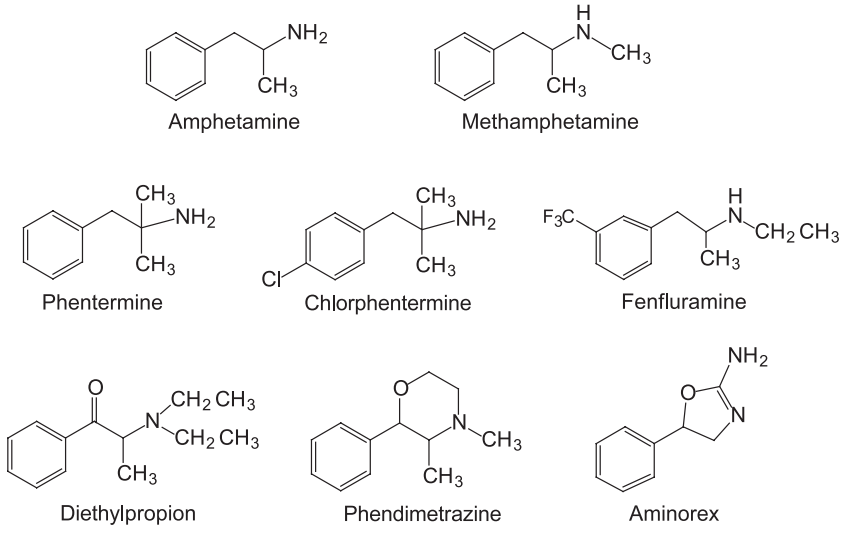

Fig. 6. Chemical structures of selected amphetamine-type appetite suppressants.

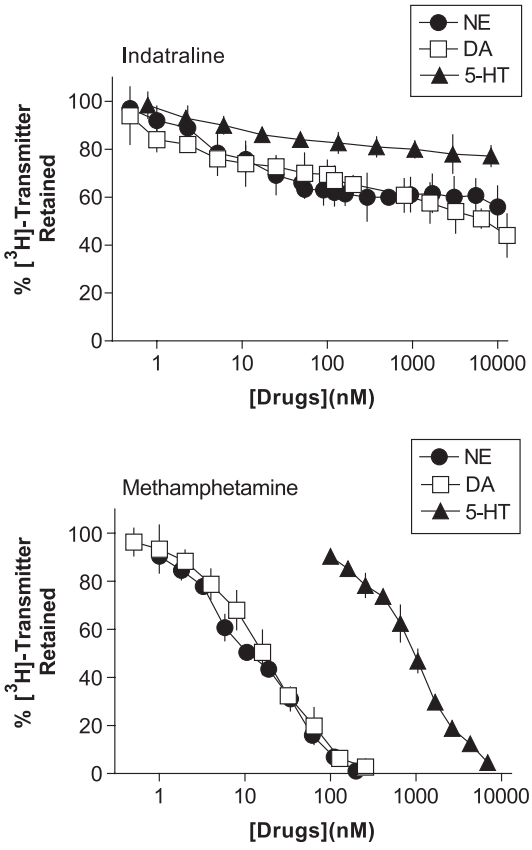

Fig. 7. Effects of the reuptake blocker indatraline (upper panel) and the transporter substrate methamphetamine (lower panel) on the release of [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]5-HT in synaptosomes. Each point is the mean ± S.D. (n = 3). Data are from Rothman et al. (2000).

porters thereby stimulating non-exocytotic transmitter release. In both cases, the immediate consequence of drug-transporter interactions is elevation of synaptic levels of dopamine, norepinephrine, and 5-HT throughout the neuraxis (Baumann et al., 1994; Kuczenski et al., 1995; Melega et al., 1995; Pepper et al., 2001). Perhaps by analogy, it is often assumed that amphetamine-type appetite suppressants act at monoamine transporters to release dopamine, norepinephrine, and 5-HT, but this hypothesis has not been rigorously tested for many anorectics. When we initially searched the biomedical literature, there was a surprising paucity of data regarding the neurochemical mechanisms underlying the actions of clinically available amphetamine-type anorectics. Thus, we began experiments to examine the molecular mechanisms associated with anorectic drugs. Because of the critical role of DAT, NET, and SERT proteins in mediating the actions of abused stimulants, we developed uptake and release assays as tools to examine the effects of anorectics on monoamine transporter function. The chief purpose of the work reviewed here is to profile the activity of various amphetamine-type appetite suppressants at DAT, NET, and SERT, in order to identify possible candidate medications for normalization therapy of stimulant dependence.

Traditionally, it has been difficult to use simple in vitro test assays to discriminate between drugs that are monoamine uptake blockers and those that are substrate-type releasers. This problem arises because both types of drugs interact with the same transporter proteins to elevate extracellular levels of neurotransmitter (Amara and Sonders, 1998; Rudnick and Clark, 1993). Our laboratory recently developed a rapid high-throughput method for measuring release of [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]5-HT from nervous tissue in vitro (Rothman et al., 2000). 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. 7, the nonselective 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]dopamine, [^{3}H]norepinephrine and [^{3}H]5-HT. Methamphetamine is much more potent at releasing dopamine and norepinephrine when compared to its effects on 5-HT, consistent with the known pharmacology of this drug (Rothman et al., 2000, 2001). The data in Fig. 8 demonstrate that low concentrations of indatraline antagonize methamphetamine-induced release of [^{3}H]dopamine, shifting the methamphetamine release curve to the right. Thus, indatraline blocks the dopamine-releasing activity of methamphetamine by binding

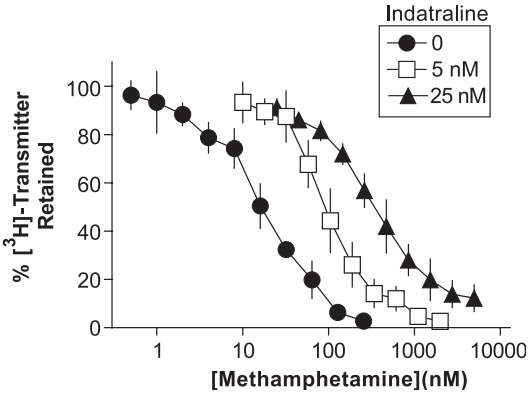

Fig. 8. Effects of indatraline (5 nM and 25 nM) on methamphetamine-evoked release of [^{3}H]dopamine. Each point is the mean ± S.D. (n = 3). Data are from Rothman et al. (2000).

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]dopamine uptake (1.9 nM). These findings show that our assay systems can 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.

Using in vitro uptake and release assays, we characterized the interaction of various transporter substrates and uptake blockers at DAT, NET, and SERT proteins. With specific reference to the dual deficit model (Fig. 5), we hoped to identify compounds which cause substrate-type release of dopamine and 5-HT with similar potency. The in vitro data are summarized in Table 10. Of the clinically available appetite suppressants, no compound releases both dopamine and 5-HT with equal potency. For example, phentermine and (+)-amphetamine are 13- and 71-fold more potent at dopamine release than 5-HT release. It is important to note that phentermine and (+)-amphetamine display their highest potency at stimulation of norepinephrine release rather than dopamine release. As described elsewhere (Yu et al., 2000), diethylpropion is totally inactive at monoamine transporters. The in vivo activity of this compound is most

Table 10
Pharmacological profile of selected agents in the dopamine, norepinephrine and 5-HT release and uptake inhibition assays

Drug	Norepinephrine release, EC_{50} (nM ± S.D.)	Norepinephrine uptake, K_i (nM ± S.D.)	5-HT release, EC_{50} (nM ± S.D.)	5-HT uptake, K_i (nM ± S.D.)	Dopamine release, EC_{50} (nM ± S.D.)	Dopamine uptake, K_i (nM ± S.D.)
Appetite suppressants and their metabolites						
Phentermine	39.4 ± 6.6	244 ± 15	3511 ± 253	13,900 ± 510	262 ± 21	1580 ± 80
(+)-Amphetamine	7.07 ± 0.95	38.9 ± 1.8	1765 ± 94	3830 ± 170	24.8 ± 3.5	34 ± 6
(–)-Ephedrine	72.4 ± 10.2	225 ± 36	>10,000	>50,000	1350 ± 124	4398 ± 213
Diethylpropion	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
N-ethylaminopropiophenone	99.3 ± 6.6	360 ± 29	2118 ± 98	3840 ± 240	>1000	>1014 ± 80
N,*N*-diethylnorpseudoephedrine	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
Phendimetrazine	8300 ± 445	>10,000	>100,000	>100,000	19,000 ± 537	>10,000
(±)-Phenmetrazine	50.4 ± 5.4	153 ± 19	7765 ± 610	>10,000	131 ± 11	607 ± 51
(+)-Phenmetrazine	37.5 ± 4.3	240 ± 24	3246 ± 263	>10,000	87.4 ± 7.8	359 ± 23
(–)-Phenmetrazine	62.9 ± 9.5	388 ± 54	>10,000	>10,000	415 ± 45	1669 ± 189
Appetite suppressants removed from clinical use						
(+)-Fenfluramine	302 ± 20	1290 ± 152	51.7 ± 6.1	150 ± 5	>10,000	22,000 ± 1100
(–)-Fenfluramine	>10,000	7187 ± 559	147 ± 19	714 ± 31	>10,000	>20,000
(±)-Fenfluramine	739 ± 57	1987 ± 205	79.3 ± 11.5	269 ± 7	>10,000	23,700 ± 1300
(±)-Norfenfluramine	168 ± 17	242 ± 25	104 ± 5	480 ± 35	1925 ± 295	4305 ± 156
(+)-Norfenfluramine	72.7 ± 5.4	205 ± 19	59.3 ± 2.4	214 ± 9	924 ± 112	2312 ± 87
(–)-Norfenfluramine	474 ± 40	2052 ± 297	287 ± 14	1175 ± 89	>10,000	19,194 ± 1048
Aminorex	26.4 ± 2.8	54.5 ± 4.8	193 ± 23	1244 ± 106	49.4 ± 7.5	216 ± 7
Chlorphentermine	>10,000	451 ± 66	30.9 ± 5.4	338 ± 6	2650 ± 273	3940 ± 110
Abused stimulants						
(–)-Methamphetamine	28.5 ± 2.5	234 ± 14	4640 ± 243	14,000 ± 644	416 ± 20	4840 ± 178
(+)-Methamphetamine	12.3 ± 0.7	48.0 ± 5.1	736 ± 45	2137 ± 98	24.5 ± 2.1	114 ± 11
(±)-MDMA	77.4 ± 3.4	462 ± 18	56.6 ± 2.1	238 ± 13	376 ± 16	1572 ± 59
Endogenous substrates						
Tyramine	40.6 ± 3.5	72.5 ± 5.0	2775 ± 234	1556 ± 95	119 ± 11	106 ± 6.0
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	6489 ± 200	86.9 ± 9.7	38.3 ± 1.6
5-HT	>10,000	3013 ± 266	44.4 ± 5.3	16.7 ± 0.9	>10,000	2703 ± 79
Transporter inhibitors						
GBR12935	>10,000	277 ± 23	>10,000	289 ± 29	>10,000	4.90 ± 0.30
GBR12909	>10,000	79.2 ± 4.9	>10,000	73.2 ± 1.5[1]	>10,000	4.3 ± 0.3
Cocaine	>10,000	779 ± 30	>10,000	304 ± 10[2]	>10,000	478 ± 25
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	5946 ± 193
Fluoxetine	>10,000	688 ± 39	>10,000	9.58 ± 0.88	>10,000	>5000
Citalopram	>10,000	4332 ± 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	0.35 ± 0.02
Indatraline	>10,000	12.6 ± 0.5	>10,000	3.10 ± 0.09	2810 ± 777	1.90 ± 0.05

From Rothman et al. (2001, 2002c). Each value is the mean ± S.D. of three experiments.

likely attributable to the *N*-deethylated metabolite, *N*-ethylaminopropiophenone (Dangor et al., 1986), which releases norepinephrine with 21-fold greater potency than it releases 5-HT, and which blocks dopamine uptake with 10-fold greater potency. Phendimetrazine, similar to diethylpropion, is inactive in the transporter assays used here. It seems probable that the activity of phendimetrazine is mediated by one or more bioactive metabolites (Beckett and Raisi, 1976). For example, it has been reported that phendimetrazine is extensively metabolized to form the *N*-demethylated metabolite, phenmetrazine. As reported elsewhere (Rothman et al., 2002c), (±)-phenmetrazine and its active isomer, (+)-phenmetrazine, are potent substrates for the norepinephrine and dopamine transporters (Table 10). The in vivo microdialysis data presented in Fig. 9 demonstrate that i.v. injections of phendimetrazine fail to alter extracellular dopamine or 5-HT in rat nucleus accumbens. In contrast, i.v. injections of equivalent doses of (+)-phenmetrazine increase extracellular dopamine in a dose-dependent manner, with a lesser effect on extracellular 5-HT. These data provide strong support for the hypothesis that phendimetrazine is a prodrug, generating phenmetrazine, a potent releaser of norepinephrine and dopamine.

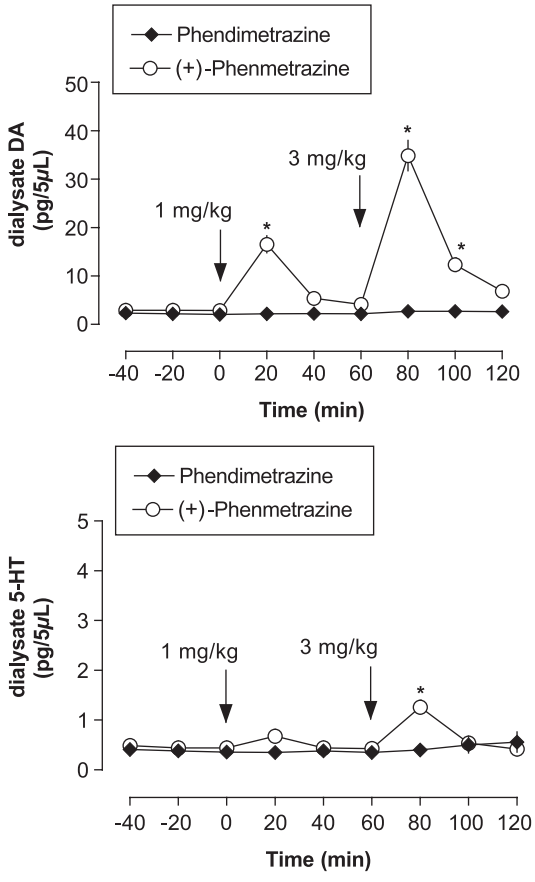

Fig. 9. Effects of i.v. phendimetrazine and (+)-phenmetrazine on extracellular dopamine (upper panel) and 5-HT (lower panel) in rat nucleus accumbens. *$p < 0.05$ compared to preinjection. Each value is the mean ± S.E.M. ($n = 6–7$ rats). From Rothman et al. (2002c).

Appetite suppressants that were once clinically available (i.e., they are now removed from the market), or were once considered for clinical use, have interesting pharmacological profiles (Table 10). (+)-Fenfluramine and (±)-fenfluramine are prototypical 5-HT releasing agents that were widely prescribed appetite suppressants, until their removal from the market due to the occurrence of cardiac valve abnormalities in some patients (Connolly et al., 1997; Connolly and McGoon, 1999). The in vitro data show that (+)-fenfluramine potently releases 5-HT, but this agent also releases norepinephrine. The narrow margin of separation between the effects of (+)-fenfluramine on SERT and NET (~5-fold) indicates that this agent probably releases both 5-HT and norepinephrine in vivo. Interestingly, fenfluramines display very low potency in assays measuring DAT activity. Recent work from our laboratory demonstrates that the *n*-deethylated metabolite of (+)-fenfluramine, (+)-norfenfluramine, is a potent norepinephrine releaser (Rothman et al., 2003). The findings depicted in Figs. 10 and 11 show the effects of (+)-fenfluramine and (+)-norfenfluramine on extracellular levels of monoamines in rat fronal cortex, as determined by in vivo microdialysis. Both drugs are powerful 5-HT releasers, producing more than 20-fold increases in dialysate 5-HT after 3 mg/kg. Additionally, both drugs are capable of increasing extracellular levels of norepinephrine and dopamine, but (+)-norfenfluramine is significantly more potent in this regard. Further experiments showed that the ability of (+)-norfenfluramine to stimulate norepinephrine and dopamine release is dependent upon interaction of the drug with NET sites in the cortex.

Aminorex, an anorectic medication that caused an epidemic of pulmonary hypertension during the 1960s (Gurtner, 1990), releases dopamine and 5-HT with similar potency. Like phentermine and (+)-amphetamine, aminorex displays its highest potency as a norepinephrine releaser. The *para*-chloro analog of phentermine, chlorphentermine, is a rather selective 5-HT releaser that blocks norepinephrine uptake and dopamine uptake at much higher doses. The in vivo microdialysis data in Figs. 12 and 13 depict the effects of selected appetite suppressants on extracellular dopamine and 5-HT in the nucleus accumbens of awake freely moving rats. Fig. 12 shows that (+)-methamphetamine and aminorex produce dose-related elevations in dialysate levels of dopamine and 5-HT. It is noteworthy that (+)-methamphetamine is at least 3-fold more potent than aminorex as a dopamine releasing agent. The fact that (+)-methamphetamine causes parallel increases in both dopamine and 5-HT is surprising based on the in vitro release data showing this drug is 30-fold selective for DATs versus SERTs. The microdialysis data demonstrate that the in vivo profile of drug-induced extracellular transmitter release may not precisely match the profile of transporter activity determined in vitro. Therefore, substrate-type releasers that appear selective for a particular transporter in vitro may not be selective when administered in the intact organism. The data in Fig. 13 illustrate that addition of a *para*-chloro group on the phenyl ring of

aminorex. Unfortunately, the clinically utility of these

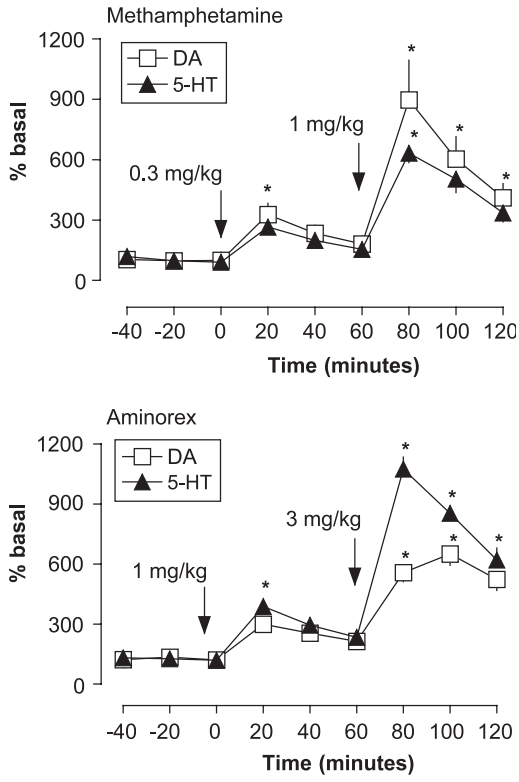

Fig. 12. Effects of (+)-methamphetamine and aminorex on extracellular dopamine and 5-HT in rat nucleus accumbens. Rats received i.v. doses of drug at time 0 and 60 min later. Dialysate samples were collected at 20-min intervals and immediately assayed for dopamine and 5-HT by HPLC-EC. Data are mean ± S.E.M. expressed as % baseline ($n = 5–6$ rats/group). From Rothman et al. (2002a).

removed from clinical use due to its association with primary pulmonary hypertension. We have recently shown that aminorex, and other amphetamine-type drugs known to increase the risk for developing primary pulmonary hypertension (fenfluramines, chlorphentermine), share the common feature of being SERT substrates (Rothman et al., 1999a). On the other hand, not all SERT substrates are associated with primary pulmonary hypertension, and we have argued that it will be possible to develop 5-HT releasing agents devoid of significant pulmonary toxicity (Rothman and Baumann, 2000). Taken together, the in vitro and in vivo findings show that amphetamine-type appetite suppressants have a wide range of activities at monoamine transporters, with each drug exhibiting its own unique profile of actions.

In previous studies, we have explored the possibility that coadministration of phentermine (a dopamine releaser) and fenfluramine (a 5-HT releaser) might be an effective strategy for treating stimulant dependence (Rothman et al., 1998a). With regard to the dual deficit model, combined administration of phentermine and fenfluramine (phentermine/fenfluramine) would be predicted to elevate extracellular levels of dopamine and 5-HT in the brain, thereby normalizing monoamine dysfunction in abstinent stimulant addicts (Baumann et al., 2000; Rothman et al., 1998a; Shoaib et al., 1997). In agreement with this notion, phentermine/(±)-fenfluramine (or phentermine/(+)-fenfluramine) reduces cocaine self-administration behavior in rats and monkeys (Glatz et al., 2002; Glowa et al., 1997). There is one report demonstrating that the phentermine/fenfluramine mixture suppresses methamphetamine self-administration in rats (Munzar et al., 1999). Perhaps more importantly, preliminary studies in humans show that phentermine/fenfluramine alleviates withdrawal symptoms in abstinent cocaine addicts, indicating that this medication could be a useful pharmacological adjunct in treatment (Hitzig, 1993; Kampman et al., 2000; Rothman et al., 1994c). Unfortunately, as mentioned above, (±)-fenfluramine and (+)-fenfluramine were removed from the market due to the occurrence of cardiac valve abnormalities in some patients taking these drugs. Currently, there are no 5-HT releasing agents available for clinical use, so the therapeutic potential of this class of drugs can no longer be evaluated in people.

Our findings with diethylpropion and phendimetrazine are particularly intriguing. Both of these anorectic medications are considered weak psychomotor stimulants due to their shared properties with illicit drugs like cocaine. For example, diethylpropion and phendimetrazine are self-administered by animals (Gotestam and Andersson, 1975;

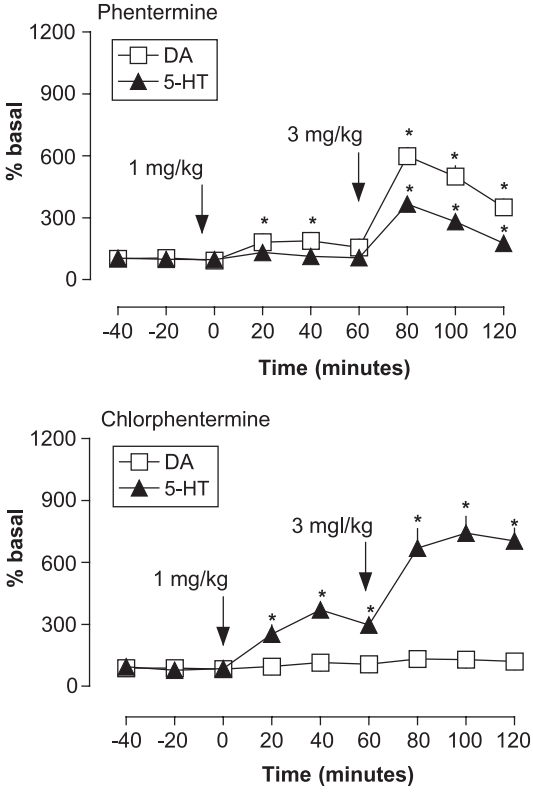

Fig. 13. Effects of phentermine and chlorphentermine on extracellular dopamine and 5-HT in rat nucleus accumbens. Rats received i.v. doses of drug at time 0 and 60 min later. Dialysate samples were collected at 20-min intervals and immediately assayed for dopamine and 5-HT by HPLC-EC. Data are mean ± S.E.M. expressed as % baseline ($n = 5–6$ rats/group). From Rothman et al. (2002a).

Griffiths et al., 1976), and both drugs exhibit discriminative stimulus properties that generalize to cocaine (Evans and Johanson, 1987; Wood and Emmett Oglesby, 1988). While it might be assumed that diethylpropion and phendimetrazine interact with monoamine transporters, our in vitro data clearly show these agents are inactive at DAT, NET and SERT. 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*-deethylated metabolite, *N*-ethylaminopropiophenone, appears to be the active metabolite since this compound has considerable activity at DAT and SERT. Unfortunately, this metabolite also has very high potency at NET, suggesting diethylpropion may not be optimally suited as a medication for stimulant dependence (see Alim et al., 1995). In the case of phendimetazine, its metabolite, phenmetrazine, potently releases dopamine and norepinephrine. The idea of using "prodrugs" as pharmacotherapies for substance use disorders deserves further study. Prodrugs might prove useful in the treatment of stimulant addictions because such agents necessarily limit the amount of active medication entering the brain. This feature of "slow kinetics" would be predicted to reduce the abuse potential of medications, especially those with strong dopamine-releasing activity.

In summary, a variety of clinical and preclinical evidence suggests that drugs capable of releasing both dopamine and 5-HT will be effective medications for agonist substitution therapy of stimulant addictions. Using simple high-throughput in vitro methods to screen the activity of compounds at monoamine transporters, we have found that none of the clinically available amphetamine-type appetite suppressants are equipotent substrates for DAT and SERT proteins. Although none of these agents are ideal, the work of Grabowski et al. (2001) showing that a slow-release formulation of (+)-amphetamine successfully treated cocaine dependence indicates that more clinical testing of anorectic agents as treatments for stimulant addictions is warranted. Future medications discovery efforts should focus on identifying new compounds which possess the desired substrate activity at DAT and SERT, but which lack the adverse effects of stimulants developed decades ago.

7. Conclusion

Psychostimulants are a fascinating group of centrally active 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 can serve as powerful drugs of abuse. The discovery of novel cocaine binding sites offers the possibility of understanding more completely the pharmacological actions of this complex drug, as well as developing more effective treatments for cocaine dependence. Similarly, a more complete understanding of the mechanism of action of older amphetamine-type anorectics will provide a basis for designing newer monoamine releasing agents with diminished adverse effects that could serve as novel treatments for stimulant dependence and other psychiatric disorders.

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