Chapter 3

Interrogating the Activity of Ligands at Monoamine Transporters in Rat Brain Synaptosomes

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Abstract

The plasma membrane transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT) are the main sites of action for therapeutic and abused stimulant drugs. As a means to identify novel medications for stimulant addiction and other psychiatric disorders, we developed in vitro assays in rat brain tissue that can be used to determine structure–activity relationships for test compounds at these monoamine transporters. Uptake inhibition assays measure the ability of drugs to block the transporter-mediated uptake of [^{3}H]neurotransmitters into synaptosomes, whereas release assays measure the ability of drugs to serve as transporter substrates that evoke efflux (i.e., release) of [^{3}H]neurotransmitters from synaptosomes by reverse transport. These assays can be used to rapidly determine the potency of test compounds at DAT, NET, and SERT under similar conditions, establishing the selectivity of drugs across all three transporters. The combined results from uptake and release assays can discriminate whether a compound is a transporter inhibitor or substrate (i.e., releaser). Our assay procedures have been used to characterize the molecular mechanism of action for older amphetamine-type medications and newer transporter ligands with therapeutic potential. The data from these assays can also predict the addictive and neurotoxic properties of abused stimulants. Information provided by these assays continues to provide insight into monoamine transporter structure and function.

Key words Transporter, Synaptosomes, Amphetamine, Stimulants, Neurotransmitter, Uptake, Release

1 Introduction

The plasma membrane neurotransmitter/sodium transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT) are the principal sites of action for medications used to treat a range of psychiatric diseases such as depression, anxiety, and attention-deficit hyperactivity disorder [1, 2]. In addition, DAT and NET are implicated in the mechanism of action of addictive stimulants like cocaine and amphetamine [3, 4]. Under normal circumstances, these monoamine transporters serve to translocate previously released neurotransmitter molecules from the extracellular medium

Heinz Bönisch and Harald H. Sitte (eds.), *Neurotransmitter Transporters: Investigative Methods*, Neuromethods, vol. 118,
DOI 10.1007/978-1-4939-3765-3_3,

back into the neuronal cytoplasm, a process known as transporter-mediated "reuptake". Drugs that interact with transporters can be divided into two classes based on their precise molecular mechanism: (1) *inhibitors*—which bind to the neurotransmitter binding site on the extracellular face of the transporter, thereby blocking the reuptake of neurotransmitters from the extracellular medium, and (2) *substrates*—which bind to the transporter and are subsequently translocated through the transporter channel into the neuronal cytoplasm, thereby triggering the efflux of intracellular neurotransmitter molecules (i.e., transporter-mediated release) [5]. Drugs that act as transporter substrates are often called "releasers" because they induce non-exocytotic transporter-mediated neurotransmitter release from neurons.

As a means to identify and characterize new medications for treating stimulant addiction and other psychiatric disorders, we sought to establish in vitro functional assays that could assess the structure–activity relationships for a large library of phenethylamine analogs at DAT, NET, and SERT. We reasoned that these assays should employ a simple and reproducible brain tissue preparation to facilitate high-throughput screening of compounds in a biologically relevant system. Additionally, we designed the assays to allow rapid assessment of potency and efficacy of drugs at all three transporters under similar conditions, and to discriminate whether test compounds act as transporter inhibitors or substrates. It is important to distinguish between transporter inhibitors and substrates because substrate drugs display a number of unique properties: they are translocated into cells along with sodium ions, they induce inward depolarizing sodium currents, and they trigger non-exocytotic release of neurotransmitters by reversing the normal direction of transporter flux (i.e., reverse transport) [6]. Finally, because substrate-type drugs are transported into the neuronal cytoplasm, they can produce intracellular deficits in monoamine neurons such as inhibition of neurotransmitter synthesis leading to long-term neurotransmitter depletions [7, 8].

The purpose of this chapter is to describe straightforward reproducible assays for measuring uptake inhibition and substrate release at DAT, NET, and SERT. All six of the assays use a crude synaptosomal preparation obtained from rat brain and share a common buffer system with only minor differences between assays. Synaptosomes are largely composed of sealed vesicle-filled nerve endings with their plasma membrane leaflets oriented in a manner akin to neurons in vivo [9, 10]. In contrast to assay systems which involve non-neuronal cells transfected with transporter proteins, synaptosomes possess all of the cellular machinery necessary for neurotransmitter synthesis, release, metabolism and reuptake. Our assays are based on the ability to detect the influence of test drugs on transporter-mediated movement of radiolabeled neurotransmitters (i.e., [^{3}H]neurotransmitters) across the synaptosomal plasma membranes.

[^{3}H]-Dopamine ([^{3}H]DA), [^{3}H]-norepinephrine ([^{3}H]NE), and [^{3}H]-serotonin ([^{3}H]5HT) are used as the radiolabeled ligands in the uptake inhibition assays for DAT, NET, and SERT, respectively. [^{3}H]-Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) is used as the radiolabeled ligand in the release assays for DAT and NET, whereas [^{3}H]5HT is used in the release assay for SERT (*see* **Note 1**). Over the years, these assays have been used to identify transporter drugs of interest that are subsequently tested using more sophisticated or labor-intensive in vivo techniques such as microdialysis, cardiovascular telemetry, intracranial self-stimulation and drug self-administration. Importantly, the pharmacological effects of drugs determined in vivo have been consistent with the drug-transporter relationships described by the uptake and release assays.

2 Materials and Preparation

2.1 Materials

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]DA, [^{3}H]NE, and [^{3}H]5HT were purchased from PerkinElmer Life Sciences (Boston, MA, USA), and [^{3}H]MPP$^+$ was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Test compounds were provided by the NIDA IRP Pharmacy (Baltimore, MD, USA), the NIDA Drug Supply Program (Rockville, MD, USA), and Research Triangle Institute (Research Triangle Park, NC, USA).

2.2 Buffers and Assay Selectivity

All uptake inhibition and substrate release assays employ the same two buffers: 0.32 M sucrose that is used for the tissue preparation and comprises 10% of the final reaction volume, and Krebs-phosphate buffer (KPB) that comprises the remaining 90% of the reaction volume. The KPB consists of the following at pH 7.4: 126 mM NaCl, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.1 mM $CaCl_2$, 0.83 mM $MgCl_2$, 0.5 mM Na_2SO_4, 11.1 mM glucose, 13.7 mM Na_2HPO_4, 1 mg/ml ascorbic acid, and 50 μM pargyline. For substrate release assays, 1 μM reserpine is added to the sucrose and KPB solutions in order to block vesicular uptake of substrates [3]. All buffers are made fresh each day. Assay selectivity is established for a single transporter of interest by adding unlabeled (i.e., nonradioactive) selective transporter blockers to the sucrose solution and KPB to prevent the interaction of radiolabeled ligands and test compounds with competing transporters (see Table 1 and **Note 2**).

2.3 Tissue Preparation

The synaptosomal preparation used in our assays is a slight modification of a procedure originally described over 50 years ago [9]. Male Sprague-Dawley rats are rendered unconscious by CO_2 narcosis, decapitated, and brains are immediately removed. Cerebella are discarded, and paired caudate-putamen are dissected and placed in 10 ml ice cold 0.32 M sucrose (up to three pairs per 10 ml) to

be used for DAT studies; the remaining brain tissue is placed in 10 ml ice cold 0.32 M sucrose (one brain per 10 ml) for use in NET and SERT studies. The tissue in sucrose is transferred to a hand-held Teflon-on-glass Potter-Elvehjem tissue grinder and gently homogenized with 12 strokes. The homogenate is centrifuged at 1000 × *g* at 4 °C, and the resulting supernatant is diluted with ice cold 0.32 M sucrose to 17.5 ml for DAT uptake, DAT release, and SERT uptake studies, or 10 ml for NET uptake, NET release, and SERT release studies; the tissue preparation is used immediately (*see* **Note 3**). Electron microscopy reveals that this crude synaptosomal tissue preparation contains intact synaptosomes, as well as myelin fragments, mitochondria, ribosomes, and vesicles [9, 10].

3 Assay Procedures

3.1 Uptake Inhibition

Uptake inhibition assays are based on the exposure of freshly prepared synaptosomes to the appropriate [^{3}H]neurotransmitter of interest (i.e., [^{3}H]DA, [^{3}H]NE, or [^{3}H]5HT). Since synaptosomal plasma membranes are sealed and intact, [^{3}H]neurotransmitter molecules added to the extrasynaptosomal medium are translocated into the intrasynaptosomal compartment by the action of membrane-bound transporter proteins. Under these conditions, transporter-mediated accumulation of [^{3}H]neurotransmitters inside the synaptosomes is linearly proportional to time (data not shown). During a typical uptake inhibition experiment, a solution containing [^{3}H]neurotransmitter and test drug is mixed with synaptosomes; this mixture is allowed to incubate for a specific period of time, within the linear portion of the [^{3}H]neurotransmitter accumulation time course. To stop the incubation, the mixture is rapidly filtered, thereby trapping accumulated intrasynaptosomal [^{3}H]neurotransmitter molecules on the filter (Whatman GF/B fiberglass filters, Brandel, Gaithersburg, MD, USA) while remaining "free" extrasynaptosomal [^{3}H]neurotransmitters are removed to waste. In this manner, the filtration process captures the [^{3}H]neurotransmitters taken up by synaptosomes during the incubation. Retained radioactivity is then quantified by liquid scintillation counting and is proportional to the quantity of synaptosomes used in the assay. If test drugs interact with monoamine transporters, the accumulation of [^{3}H]neurotransmitter inside of the synaptosomes (i.e., uptake) is reduced because the test drug and [^{3}H]neurotransmitter molecules compete for the same binding site on the transporter protein. This degree of "uptake inhibition" is proportional to the concentration of test drug; the concentration of test drug required to inhibit uptake by 50% (IC_{50}) defines the potency of the uptake inhibitor for the transporter.

We first used this technique to examine the transporter selectivity of a homolog of the DAT blocker GBR12935, LR1111 [11],

and continue to use the method with only minor modifications [12]. Uptake inhibition assays are conducted in triplicate at 25 °C (DAT and SERT) or 37 °C (NET) in polystyrene test tubes (12×75 mm). Assays are initiated by the addition of 100 μl of freshly prepared synaptosomes to 900 μl KPB that contains the appropriate [^{3}H]neurotransmitter, selective blockers (see Table 1), and test drug. With regard to radiolabeled transmitter concentrations, we use final concentrations of 5 nM [^{3}H]DA, 10 nM [^{3}H]NE and 5 nM [^{3}H]5HT for the DAT, NET, and SERT uptake inhibition assays, respectively. Test drug solutions are prepared in KPB containing 1 mg/ml bovine serum albumin over a range of eight different doses in order to construct an inhibition curve that establishes an IC_{50} value for the test drug. Nonspecific uptake is measured by incubating synaptosomes in the presence of a large excess (1 μM) of the nonselective uptake inhibitor indatraline. The reactions are stopped after 15 min (DAT), 10 min (NET), or 30 min (SERT) by rapid vacuum filtration with a cell harvester (Brandel, Gaithersburg, MD, USA) over GF/B filter paper presoaked in wash buffer maintained at 25 °C (10 mM Tris-HCl, pH 7.4/150 mM NaCl). Filters are rinsed with 6 ml wash buffer, and retained tritium is quantified by a MicroBeta 2 liquid scintillation counter (PerkinElmer, Boston, MA, USA) after overnight extraction in 0.6 mL of liquid scintillation cocktail (Cytoscint, MP Biomedicals, Santa Ana, CA, USA).

In order to describe the method for calculating the uptake IC_{50}, the following definitions are necessary:

Total Uptake (TU) = cpm accumulated in the absence of any drug.

Nonspecific Uptake (NS) = cpm accumulated in the presence of total transporter blockade using1 μM indatraline.

Table 1
Uptake inhibitors used to establish assay selectivity

Assay	Desipramine (blocks NET)	Citalopram (blocks SERT)	GBR 12935 (blocks DAT)	Nomifensine (blocks NET)
DAT uptake[a]				
SERT uptake			50 nM	100 nM
NET uptake[b]			50 nM	
DAT release	100 nM	100 nM		
SERT release			50 nM	100 nM
NET release		100 nM	50 nM	

Selective uptake inhibitors are added in the indicated concentrations to both the sucrose solution and KPB
[a]DAT uptake selectivity is established by surgical isolation of the caudate-putamen, a region so enriched in DAT that measurable uptake of [^{3}H]DA by NET or SERT does not occur; selective blockade of NET or SERT is not required
[b]SERT affinity for [^{3}H]NE is so weak that SERT blockade is not required

Maximal uptake inhibition (MU) = TU – NS.

Specific uptake inhibition (SU) = (cpm in the presence of test drug) – NS.

% uptake inhibition = 100 – SU/MU × 100.

The data from three experiments, expressed as % uptake inhibition, are then fit to a dose-response curve equation: $Y = I_{max} \times ([D]/([D] + IC_{50}))$ for the best fit estimates of the I_{max} and IC_{50}, where [D] is the concentration of test drug. Over the years, we have used a number of different commercially available software programs for data analysis and curve fitting, including MLAB-PC (Civilized Software, Silver Spring, MD, USA), KaleidaGraph (Synergy Software, Reading, PA, USA), and Prism (GraphPad Software, La Jolla, CA, USA).

Similar to uptake inhibitors, transporter substrates will compete with [^{3}H]neurotransmitter molecules to occupy transporter binding sites during uptake inhibition assays. Additionally, substrate drugs will evoke efflux of any accumulated transmitter. The combined effects of substrate drugs will reduce the amount of [^{3}H]neurotransmitter accumulated into synaptosomes in the uptake inhibition assays. Thus, uptake inhibition assays cannot discriminate whether transporter drugs are inhibitors or substrates. Nevertheless, our initial experiments demonstrated that a number of known transporter substrates (e.g., amphetamine-related compounds) were generally three- to tenfold more potent in substrate release assays when compared to their potency in the uptake inhibition assays (see Table 2) [3]. This pattern has persisted with nearly all substrate drugs that we have tested in the uptake inhibition assays [13, 14].

3.2 Substrate Release

Using the uptake inhibition assay conditions described above, synaptosomes will accumulate [^{3}H]neurotransmitter molecules in a biphasic manner, with an initial linear burst followed by a slower phase that does not plateau. The initial phase of [^{3}H]neurotransmitter uptake is mediated by the action of plasma membrane monoamine transporters (i.e., DAT, NET and SERT), whereas the later phase is related to the uptake of neurotransmitters into synaptic vesicles by the action of the vesicular monoamine transporter-2 (VMAT-2). Inclusion of 1 μM reserpine, an irreversible blocker of VMAT-2, in the sucrose and KPB solutions allows for the steady state accumulation of [^{3}H]neurotransmitter molecules into the intrasynaptosomal compartment within 60 min, by preventing the uptake of [^{3}H]neurotransmitter and substrate drugs into synaptic vesicles that are present in our tissue preparation [3]. Thus, the inclusion of reserpine in the sucrose and KPB removes any effects of VMAT-2 from the assay system.

The substrate release assay is based on the efflux of previously accumulated (i.e., preloaded) synaptosomal [^{3}H]neurotransmitter by exposure to test substrates, via a transporter-mediated exchange

Table 2
Pharmacological profile of selected agents in transporter release and uptake inhibition assays[a]

	DAT release	DAT uptake	NET release	NET uptake	SERT release	SERT uptake
Drug	**EC_{50} (nM ± SD)**	**IC_{50} (nM ± SD)**	**EC_{50} (nM ± SD)**	**IC_{50} (nM ± SD)**	**EC_{50} (nM ± SD)**	**IC_{50} (nM ± SD)**
Aminorex	49.4 ± 7.5	216 ± 7	26.4 ± 2.8	54.5 ± 4.8	193 ± 23	1244 ± 106
Phentermine	262 ± 21	1580 ± 80	39.4 ± 6.6	244 ± 15	3511 ± 253	13,900 ± 510
(+)-Amphetamine	24.8 ± 3.5	34.0 ± 6.0	7.07 ± 0.95	38.9 ± 1.8	1765 ± 94	3830 ± 170
(−)-Methamph	416 ± 20	4840 ± 178	28.5 ± 2.5	234 ± 14	4640 ± 243	14,000 ± 644
(+)-Methamph	24.5 ± 2.1	114 ± 11	12.3 ± 0.7	48.0 ± 5.1	736 ± 45	2137 ± 98
(+)-Fenfluramine	>10,000	22,000 ± 1100	302 ± 20	1290 ± 152	51.7 ± 6.1	150 ± 5
(±)-Fenfluramine	>10,000	23,700 ± 1300	739 ± 57	1987 ± 205	79.3 ± 11.5	269 ± 7
(−)-Ephedrine	1350 ± 12	4398 ± 213	72.4 ± 10.2	225 ± 36	>10,000	>50,000
Tyramine	119 ± 11	106 ± 6	40.6 ± 3.5	72.5 ± 5.0	2775 ± 234	1556 ± 95
(±)-MDMA	376 ± 16	1572 ± 59	77.4 ± 3.4	462 ± 18	56.6 ± 2.1	238 ± 13
Norepinephrine	869 ± 51	357 ± 27	164 ± 13	63.9 ± 1.6	>10,000	>50,000
Dopamine	86.9 ± 9.7	38.3 ± 1.6	66.2 ± 5.4	40.3 ± 4.4	>10,000	6489 ± 200
5-HT	>10,000	2703 ± 79	>10,000	3013 ± 266	44.4 ± 5.3	16.7 ± 0.9
GBR12935	>10,000	3.70 ± 0.40	>10,000	277 ± 23	>10,000	289 ± 29
Mazindol	>10,000	25.9 ± 0.6	>10,000	2.88 ± 0.17	>10,000	272 ± 11
Desipramine	>10,000	5946 ± 193	>10,000	8.32 ± 1.19	>10,000	350 ± 13
Fluoxetine	>10,000	>5000	>10,000	688 ± 39	>10,000	9.58 ± 0.88
Citalopram	>10,000	20,485 ± 923	>10,000	4332 ± 295	>10,000	2.40 ± 0.09
RTI-55	>10,000	0.83 ± 0.09	>10,000	5.89 ± 0.53	>10,000	1.00 ± 0.03
Indatraline	2810 ± 777	1.90 ± 0.05	>10,000	12.6 ± 0.5	>10,000	3.10 ± 0.09

Each value is the mean ± SD of three experiments

[a]The data in this table were reported in ref. [3]

process thought to involve the reversal of normal transporter flux (i.e., "reverse" transport) [15, 16]. Substrate-type drugs will deplete [^{3}H]neurotransmitter from synaptosomes via this reverse transport mechanism in a concentration-dependent manner; the amount of test drug required to release 50% of the preloaded [^{3}H] neurotransmitter (EC_{50}) defines the potency of that substrate for the transporter. Similar to the uptake inhibition assay, transporter selectivity in the substrate release assay is achieved by inclusion of unlabeled selective uptake inhibitors in the sucrose solution and KPB to prevent the interaction of [^{3}H]neurotransmitters and test compounds with competing transporters (see Table 1). For the SERT release assay, [^{3}H]5HT is used as the radiolabeled neurotransmitter. [^{3}H]MPP^+ is used for the DAT and NET release assays because it is less diffusible across cell membranes and produces a better signal-to-noise ratio than [^{3}H]DA and [^{3}H]NE.

Prior to the release assay, synaptosomes must be preloaded with [^{3}H]neurotransmitters. To accomplish this step, synaptosomes are incubated with the appropriate [^{3}H]neurotransmitter in the presence of selective uptake inhibitors (see Table 1) in KPB containing 1 μM reserpine. The incubation is allowed to reach equilibrium (1 h at 25 °C). To initiate the release reaction, 850 μl of synaptosomes preloaded with [^{3}H]neurotransmitter are added to 12 × 75 mm polystyrene test tubes that contain 150 μl of test drug that has been diluted in KPB containing 1 mg/ml bovine serum albumin. Each test condition in the release assay is run in triplicate. After 30 min (DAT, NET) or 5 min (SERT), the release reactions are stopped using a cell harvester (Brandel), by rapid vacuum filtration over GF/B filter paper presoaked in wash buffer maintained at 25 °C (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). For the NET release assay, the wash buffer contains 2% polyethylenimine to minimize nonspecific adsorption of [^{3}H]MPP^+ to the filter paper. Filters are rinsed with 6 ml wash buffer, and retained tritium is quantified by a MicroBeta 2 liquid scintillation counter after overnight extraction in 0.6 mL of Cytoscint. For the release assay, it is important to note that the amount of tritium retained is inversely proportional to the extent of release from synaptosomes; that is, a lower amount of retained tritium reflects a higher degree of transporter-mediated release.

In order to describe the method for calculating the release EC_{50}, the following definitions are necessary:

Basal "Leak" Release (BLR) = cpm in the absence of any drug.

Total Evoked Release (TER) = cpm in the presence of saturating concentrations of the nonspecific releaser, tyramine—10 μM tyramine for DAT and NET assays, or 100 μM tyramine for SERT assays.

Maximal release (MR) = BLR – TER.

Specific release (SR) = (cpm in the presence of test drug) – TER.

% maximal release = 100 – SR/MR × 100.

The data from three experiments, expressed as % maximal release, are then fit to a dose-response curve equation: $Y = E_{max} \times ([D]/([D] + EC_{50}))$ for the best fit estimates of the E_{max} and EC_{50}, where [D] is the concentration of test drug. Data analysis and curve fitting can be accomplished using the same software programs noted above for the uptake inhibition assays.

In the substrate release assays, test drugs are not exposed to synaptosomes until the synaptosomes have been preloaded with [^{3}H]neurotransmitter. Subsequent exposure of preloaded synaptosomes to test drugs that are substrates provokes the depletion (i.e., release) of [^{3}H]neurotransmitter from the synaptosomes, via transporter-mediated exchange, in a concentration-dependent manner [17, 18]. Importantly, uptake inhibitors show no or minimal activity in substrate release assays because they are not transported by DAT, NET, and SERT and cannot evoke release of [^{3}H] neurotransmitter from preloaded synaptosomes (see Table 2). Therefore, unlike the uptake inhibition assays, the substrate release assays are able to discriminate between drugs that are uptake inhibitors and drugs that are substrates at transporters.

4 Summary

The uptake inhibition and substrate release assays provide straightforward, relatively high-throughput methods for assessing the interaction of drugs with the monoamine transporter proteins for dopamine, norepinephrine and serotonin. We use a crude synaptosomal tissue preparation that is simple to generate, yet provides highly reproducible results. These assays allow for the determination of relative potencies of drugs at DAT, NET, and SERT, and are able to distinguish drugs that are transporter uptake inhibitors from those that are transporter substrates. Data from the combined assay results are used to identify drugs of interest that can be further evaluated by in vivo techniques such as microdialysis, cardiovascular telemetry, intracranial self-stimulation and drug self-administration.

We have used the uptake inhibition and substrate release assays to reexamine the mechanism of action for anorectic medications that were marketed in the 1970s, such as fenfluramine and diethylpropion. For fenfluramine, we found that the parent compound and its *N*-dealkylated metabolite, norfenfluramine, display potent substrate activity at NET, as well as their established substrate activity at SERT [19]. The SERT substrate activity of fenfluramine and norfenfluramine has been linked to adverse effects such as primary pulmonary hypertension. In the case of diethylpropion, it was discovered that the parent compound is devoid of transporter

activity while the *N*-dealkylated metabolite, *N*-ethylcathinone, is active as a DAT inhibitor and SERT substrate [20]. Thus, diethylpropion is a prodrug which requires hepatic biotransformation to *N*-ethylcathinone to exert its therapeutic actions.

Testing large libraries of compounds in our assays has led to the identification of ligands with novel profiles of action at monoamine transporters. For example, most transporter inhibitors competitively block the uptake of [^{3}H]neurotransmitter by binding to the orthosteric site on the transporter protein, but we have discovered quinazolinamine analogs which noncompetitively inhibit uptake at DAT, NET, and SERT [12]. These allosteric modulators potently inhibit the uptake of [^{3}H]neurotransmitter without substantially affecting transporter ligand binding or amphetamine-induced release. Such results suggest that these compounds interact with monoamine transporters in a manner which selectively interrupts the uptake portion of the transport cycle. In other studies, we have identified unique transporter substrates which evoke only 50–70% of the maximal release response, indicative of "partial" releasing ability [21]. The molecular mechanism responsible for partial release is unknown and under investigation, but these compounds blur the distinction between "pure" uptake inhibitors and "pure" substrate-type releasers. From a drug development perspective, allosteric modulators and partial substrates at monoamine transporters may represent promising new leads for medication discovery.

Recently, we have used the uptake inhibition and substrate release assays to determine the molecular mechanism of action for synthetic cathinone compounds that are the active ingredients in psychoactive "bath salts" products [14, 22]. These products are used as substitutes for more traditional stimulant drugs of abuse such as cocaine and amphetamine. In initial experiments, we demonstrated that 3,4-methylenedioxypyrovalerone (MDPV) is a potent uptake inhibitor at DAT and NET, whereas 4-methylmethcathinone and 3,4-methylenedioxymethcathinone are nonselective transporter substrates [14, 22]. Subsequent structure–activity studies revealed that other cathinone analogs which possess an *N*-containing pyrrolidine ring, similar to MDPV, act as catecholamine-selective inhibitors, probably because these compounds are sterically too large to permeate the transporter channel as substrates [23, 24]. Phenyl ring-substitutions can markedly influence the transporter selectivity for cathinone compounds, such that bulky *para* substituents engender increased potency towards SERT versus DAT [25, 26]. A shift in selectivity towards SERT over DAT reduces the abuse liability of cathinones, similar to the findings with ring-substituted amphetamines [7, 25]. Results from our uptake and release assays have been used to explore the interaction of cathinone compounds with transporters at the molecular level, using models of DAT and SERT based on the reported crystal structures of bacterial LeuT and *Drosophila* DAT proteins [23, 26]. It is hoped

that new research findings generated from our in vitro assays will continue to provide insights into the structure and function of monoamine transporter proteins.

Notes

1. [^{3}H]DA, [^{3}H]NE, [^{3}H]5HT, and [^{3}H]MPP^+ are stored at –80 °C in their original source vials and are thawed on the day of assay at room temperature. Every effort is made to minimize the amount of time that [^{3}H]neurotransmitters in their source vials are in the thawed liquid state, in order to minimize chemical degradation. Aliquoting to eliminate degradation related to repeated freeze–thaw cycles does not improve the stability of the radioligands, as measured by the observed signal-to-noise ratio of each assay.
2. All buffers are freshly made each day. To simplify the procedure, the following KPB components are combined in a partial 10× stock solution (10× KPB) that is aliquoted, stored frozen, and used as needed: NaCl, KCl, KH_2PO_4, $CaCl_2$, $MgCl_2$, and Na_2SO_4. To prepare KPB, a 30 ml aliquot of 10× KPB is thawed and diluted to 300 ml with water. Ascorbic acid (300 mg), 0.585 g Na_2HPO_4, 0.6 g dextrose, and 3 mg pargyline are added, and the pH is adjusted to 7.4 with drop wise 2 N NaOH. Appropriate selective blockers (and reserpine for release assays) are then added (see Table 1). Seventy-five milliliters of the KPB is used for drug dilutions, and bovine serum albumin is added to this solution to yield a final concentration of 1 mg/ml.
3. Synaptosomes are prepared each day from freshly excised rat brain and are used immediately after the centrifugation step to initiate the uptake inhibition and substrate release assays. Under these conditions the synaptosomes are able to maintain [^{3}H] neurotransmitter equilibrium for up to 4 h.

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