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Effects of *N*-alkyl-4-methylamphetamine optical isomers on plasma membrane monoamine transporters and abuse-related behavior

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Abstract

4-Methylamphetamine (4-MA) is an emerging drug of abuse that acts as a substrate at plasma membrane transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), thereby causing non-exocytotic release of monoamine transmitters via reverse transport. Prior studies by us showed that increasing the *N*-alkyl chain length of *N*-substituted 4-MA analogs converts 4-MA from a transportable substrate (i.e., releaser) at DAT and NET to a non-transported blocker at these sites. Here we studied the effects of the individual optical isomers of *N*-methyl-, *N*-ethyl-, and *N*-*n*-propyl 4-MA on monoamine transporters and abuse-related behavior in rats because action/function might be related to stereochemistry. Uptake inhibition and release assays were conducted in rat brain synaptosomes whereas electrophysiological assessments of drug-transporter interactions were examined using cell-based biosensors. Intracranial-self stimulation (ICSS) in rats was employed to assess abuse potential *in vivo*. The experimental evidence demonstrates that *S*(+)-*N*-methyl 4-MA is a potent and efficacious releaser at DAT, NET, and SERT with the highest abuse potential among the test drugs whereas *R*(-)-*N*-methyl 4-MA, is a less

Contributions: RAG proposed the project. UB synthesized the various optical isomers under the direction of RAG. DW measured synaptosomal uptake and release under the supervision of MHB. JE oversaw the calcium assays conducted by AH, RS and IR. SN oversaw the ICSS assays conducted by FS. All co-authors have reviewed the draft manuscript and had an opportunity to contribute to its preparation.

COI: None of the author of this manuscript have any COI to report.

Supporting Information:

Concentration-response plots of neurotransmitter inhibition and release using synaptosomes for isomers of **3–5**, and their ability to evoke or block Ca^{2+} signals in transporter-expressing cells, and normalized Ca^{2+} signals for **3S** and **4S** at DAT versus time Figures S1–S4). A comparison of the effect of racemic mephedrone (**2**) and **3** on transporter-mediated uptake and release in rat brain synaptosomes (Table S1).

potent releaser with reduced abuse potential. The *S*(+)ethyl analog displays decreased efficacy as a releaser at DAT but retains full release activity at NET and SERT with a reduction in abuse-related effects; the *R*(-)ethyl analog has a similar profile but is less potent. *S*(+)-*N*-Propyl 4-MA is a non-transported blocker at DAT and NET, but an efficacious releaser at SERT, whereas the *R* enantiomer is almost inactive. In conclusion, the *S* enantiomers of the *N*-alkyl 4-MA analogs are most potent. Lengthening the *N*-alkyl chain converts compounds from potent non-selective releasers showing abuse-related effects, to more selective SERT releasers with no apparent abuse potential.

Keywords

Methedrone; Amphetamine; Dopamine transporter (DAT); Norepinephrine transporter (NET); Serotonin transporter (SERT); Drug abuse; Synthetic cathinones; Bath Salts; ICSS

Introduction

Cathinone (**1**) and mephedrone (**2**) are stimulant-type drugs of abuse that are under Schedule I control in the United States. From a structural perspective, cathinone is the β -keto analog of amphetamine whereas mephedrone is the β -keto analog of 4-methyl-*N*-methylamphetamine (*N*-methyl 4-MA; **3**). The pharmacology of cathinone (**1**) and mephedrone (**2**) has been extensively investigated (reviewed^{e.g.} 1,2) whereas much less is known about **3**.

Compound **3** is the *N*-methyl counterpart of 4-methylamphetamine (4-MA). 4-MA was initially studied in the 1950s as an anorectic agent,³ but never achieved widespread clinical use. 4-MA made its debut as an obscure “recreational drug” in the U.S. in 1973 and in Europe in 1989, but it was not until twenty years later that it began to attract the attention of law enforcement agencies as a popular drug of abuse.^{4–6} It is noteworthy that 4-MA appeared on the recreational drug market at about the same time as did its β -keto analog mephedrone (**2**).^{5,6} 4-MA exerts its pharmacological effects as a *non-selective* substrate (i.e., releaser) at plasma membrane transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters, thereby causing non-exocytotic release of monoamine neurotransmitters by reverse transport.^{7,8}

Our laboratories and others previously reported that mephedrone (**2**) is also a non-selective releaser at DAT, NET, and SERT^{9,10,11–13} that mimics the established molecular mechanism

of action for 3,4-methylenedioxy-*N*-methylamphetamine (MDMA). The two key structural differences between 4-MA and mephedrone (**2**) are the presence of the β -keto group and an *N*-methyl substituent of the latter that is not present in the former. Compound **3** represents the *des*- β -keto analog of **2**. Table S1 (SI) provides a potency comparison between **3** and mephedrone (**2**) on transporter-mediated uptake and release in rat brain synaptosomes. There is generally a <2-fold difference in their potency given that the β -keto group is the only structural difference between the two agents.

Structure-activity studies show that increasing the bulk (i.e., size, length?) of the terminal amine substituent of cathinone or amphetamine-related analogs converts them from substrate-type releasing agents at DAT to non-transported blockers that inhibit dopamine uptake.^{e.g. 1,2,14} We recently investigated the effects of increasing the *N*-alkyl chain length on the transporter activity of 4-MA.¹⁵ Specifically, we examined compounds **3–6** that introduced linear bulk on the amine of 4-MA in a homologous manner. That is, all of these compounds were secondary amines, but bear amine substituents with increasing steric bulk (length). In general, within this series, increased bulk/length of the *N*-alkyl substituent was associated with varied potency to inhibit uptake at brain DAT, NET, and SERT in rat brain synaptosomes, decreased potency as substrates, and reduced efficacy to promote release.¹⁵ For example, only **3**, as with **2**, was fully efficacious as a releasing agent at DAT whereas **4** and **5** were partial releasers.¹⁵ In contrast, **3–5** were fully efficacious releasing agents at SERT. Compound **6** did not display releasing action at DAT, NET, or SERT, was a very weak uptake inhibitor at DAT and NET, and inactive at SERT.¹⁵

Although synaptosome assays can distinguish fully efficacious releasers from transport blockers, they are less reliable for discriminating the molecular mechanisms of action for compounds inducing low-efficacy partial release (i.e., “partial” releasing agents).¹⁵ As a means to investigate the mechanism of partial releasers in greater detail, we developed methods that involved co-transfection of cells with DAT, NET, and SERT along with voltage-gated calcium channels. Under these circumstances, the electrophysiological effects of transporter substrates or blockers are transduced to measurable calcium fluorescence. Using calcium flux assays, it was shown that compounds **4** and **5** act as uptake inhibitors at DAT, but as releasers at SERT.¹⁵ That is, the latter compounds displayed hybrid transporter activity which was characterized by uptake blockade at DAT but substrate-type release at SERT.

3 R = CH₃

4 R = CH₂CH₃

5 R = CH₂CH₂CH₃

6 R = CH₂CH₂CH₂CH₃

In our previous work, compounds **3–6** were examined as racemic mixtures.¹⁵ Given the transporter profiles shown by the amine-substituted 4-MA racemates,¹⁵ here we synthesized and examined the transporter activity of their individual optical isomers to determine if their actions are *i*) stereoselective, *ii*) stereospecific, or, more intriguingly, *iii*) unique (i.e., one effect, for example, release, is related primarily to one optical isomer whereas the other action, i.e., uptake inhibition, is related to its opposite enantiomer). Data from these experiments could aid in the understanding of why “partial release” was observed with the racemates. Hitherto, such a study has not been conducted on these types of analogs. Hence, we prepared the individual optical isomers of **3–5** for evaluation. Due to the low potency of compound **6** as determined previously,¹⁵ the optical isomers of *N*-*n*-butyl 4-MA (**6**) were not evaluated. The isomers were also examined for their likely abuse potential in intracranial self-stimulation (ICSS) studies with rats.

Results

Synthesis

Several methods have been reported for the chiral resolution/separation of racemic 4-MA-like phenylisopropylamines; however, each compound typically requires unique conditions and overall yields are often quite low.^{16,17} Recently, we described the synthesis of different enantiomerically pure phenylisopropylamines employing a well-established synthetic route via the commercially available chiral α -methylbenzylamine.¹⁸ A phenyl-2-propanone can be converted in a pair of epimeric *N*-(α -phenethyl)phenylisopropylamines by a reductive amination reaction. Separation of the two epimers by crystallization or chromatography,

followed by de-benzylation affords the enantiomerically pure phenylisopropylamines. But, for phenylisopropylamines requiring commercially unavailable phenyl-2-propanones, this method is time-consuming and tedious. Attempts were made using this synthetic route but the results were not satisfactory. So, for these reasons, an alternative stereoselective approach was selected by taking advantage of the thermodynamic stability of propylenebenzenium ions.¹⁹ 4-(Methylphenyl)magnesium bromide was reacted with CuI and commercially available chiral propylene oxides, promoting stereoselective ring opening of the latter, to give **7S** and **7R** (Scheme 1). Reaction of the resultant alcohols with MsCl and Et₃N afforded compounds **8S** and **8R** in good yield which, upon treatment with the selected amine in dimethylformamide, furnished compounds **3-5R** and **3-5S** through an S_N2 displacement of the leaving group and consequent inversion of configuration of the chiral center.²⁰

Transporter-mediated uptake inhibition and release in synaptosomes

The pharmacological activity of *N*-alkyl 4-MA enantiomers at monoamine transporters was studied using rat brain synaptosomes. Uptake inhibition assays using this preparation determine if compounds can block the accumulation of radiolabelled neurotransmitters into synaptosomes by transporter-mediated mechanisms (Table 1 and Figure S1). In addition, compounds were tested in separate synaptosome assays that measure the ability of compounds to evoke transporter-mediated release from synaptosomes preloaded with a radioactive substrate (release assay) (Table 2 and Figure S2).

Uptake inhibition studies showed that **3S** and **3R** were fully efficacious blockers of neurotransmitter uptake at DAT, NET, and SERT, but **3S** was more potent than **3R** (Table 1). Complementary release assays demonstrated that these two compounds behave as potent, fully efficacious releasers indicative of substrate activity (Table 2). Compound **3S** was the most potent enantiomer at DAT and SERT, whereas **3S** and **3R** were nearly equipotent at NET (Table 2).

Although compounds **4S** and **4R** were fully efficacious blockers on neurotransmitter uptake at DAT, NET, and SERT (Table 1), they showed only ~50% efficacy in DAT release assays indicating partial release activity (Table 2). In contrast, these compounds showed full release activity at NET and SERT (Table 2), suggesting substrate activity at these two transporters. At all transporters **4S** was more potent than **4R** in uptake inhibition and release assays (Tables 1 and 2).

When compounds **5S** and **5R** were examined in uptake inhibition assays, both were fully efficacious blockers of neurotransmitter uptake at DAT, NET, and SERT, but their effects were much weaker than isomers of **3** or **4**. Compound **5S** was inactive as a releasing agent at DAT and NET, but showed fully efficacious release activity at SERT, indicating this compound is a blocker at DAT and NET, and a substrate at SERT; **5R** was inactive in the release assay for DAT and NET and showed negligible release at high concentrations at SERT, supporting its role as blocker at all transporters.

A common theme for this set of compounds in the synaptosome assays is that the *S* enantiomer is considerably more potent than *R*. The only exception is **3S** and **3R** that

showed similar potency as releasers at NET. In addition, a general trend is that the elongation of the N-alkyl chain is accompanied by a gradual decrease in potency to block uptake at all transporters. Another important finding is that the gradual transition from N-methyl, N-ethyl, to N-propyl, corresponds to releaser, partial releaser, to blocker at DAT, respectively, whereas this transition corresponds to releaser, releaser, to blocker at NET. In the SERT assays, all *S* enantiomers of the series function as releasers. For the *R* enantiomers, the N-methyl and N-ethyl compounds are clear releasers at SERT, and the N-propyl substituted compound showed very weak partial release activity more consistent with a blocker.

Calcium fluorescence assay

To further characterize the molecular mechanism of action for *N*-alkyl 4-MA enantiomers on monoamine transporters, we examined the effects of the compounds in HEK cells co-expressing monoamine transporters and voltage-gated Ca^{2+} channels. It is well accepted that the interaction of substrates with monoamine transporters induces inward depolarizing currents mediated by co-transport of sodium ions with substrate molecules.^{21–23} In our calcium fluorescence assay, substrate-induced transporter-mediated currents are coupled to voltage-gated Ca^{2+} channels which causes opening of calcium channels increasing the intracellular Ca^{2+} concentration.^{15,24,25} As we have described before¹⁵ and as shown here, cells expressing these two proteins and loaded with a cytosolic Ca^{2+} sensor constitute a biosensor that produces a detectable Ca^{2+} signal in response to transporter-mediated currents induced by substrates (Figure. S3A). On the other hand, inhibitors of monoamine transporters can be studied by competing the Ca^{2+} signal induced by a fixed concentration of a known substrate (Figure. S3B). This technique is powerful for identifying hybrid compounds defined as those acting as substrates at a subset of transporters while acting as blockers at others.^{15,26}

Compounds **3S** and **3R** produced Ca^{2+} signals in cells expressing DAT, NET or SERT, indicating that they generate inward currents and suggesting that they behave as substrates at these three transporters. Compounds **4S** and **4R** were hybrid compounds; in DAT-expressing cells, although they do not induce Ca^{2+} signals, they blocked dopamine-induced Ca^{2+} signals supporting their role as blockers. In contrast, these two compounds generated Ca^{2+} signals in NET- and SERT-expressing cells revealing them as substrates at these transporters. Compound **5S** was also a hybrid compound that has a blocker profile at DAT and NET, but substrate profile at SERT. Compound **5R** was very weak and acted as a blocker on DAT and NET. In cells expressing SERT, **5R** produced small Ca^{2+} signals that were reliable only at 100 μM concentration, but it partially blocked the 5-HT-induced Ca^{2+} signal in the 5 μM IC_{50} range, suggesting that it works as a partial substrate at SERT.

For nearly all the compounds tested, the *S* isomers showed significantly higher potency than their corresponding *R* enantiomers at monoamine transporters (Table 3, Figure S4), suggesting that the action of the racemates¹⁵ resides primarily with the *S* enantiomer. The exceptions are compounds **3S** and **3R** which were nearly equipotent in cells expressing NET. This equipotent interaction with NET is gradually diminished when the N-alkyl chain is elongated. In addition, there is a trend along the 4-MA scaffold that gradual extension the

N-alkyl chain progressively decreases the potency of substrates or blockers to produce or inhibit Ca^{2+} signals in cells expressing monoamine transporters.

The efficacy of compounds to generate or block Ca^{2+} signals was measured and the results are shown in Table 4. For substrates, 100% efficacy is defined as the Ca^{2+} signal amplitude evoked by the corresponding endogenous neurotransmitter (DA, NE, or 5-HT), and for blockers 100% efficacy is the complete blockade of the Ca^{2+} signal produced by a fixed concentration of the endogenous substrate. Compounds **3S** and **3R** showed >90% efficacy producing Ca^{2+} signals in cells expressing DAT, NET, or SERT. Compound **4S** and **4R** also produced >90% efficacy blocking the Ca^{2+} signal on DAT-cells or evoking Ca^{2+} signals in SERT-cells, respectively. In NET cells, these compounds produced Ca^{2+} signals with diminished efficacy, **4S** and **4R** only produce 57% and 35% of the NE-associated Ca^{2+} signal amplitude, respectively. To improve the dynamic range of these recordings the experiments were repeated using a different Ca^{2+} channel, $\text{Ca}_v1.3$, that has a lower threshold of activation and requires less depolarization to open.²⁵ When $\text{Ca}_v1.3$ rather than $\text{Ca}_v1.2$ was used, the efficacy of **4S** and **4R** to induce Ca^{2+} signals increased to 88% and 68%, respectively. The improvement of the signal, when $\text{Ca}_v1.3$ was used, suggests that **4S** and **4R** act as partially efficacious substrates to evoke modest inward current at NET. Compound **5S** blocked most of the signal in cells expressing DAT or NET evoked by DA or NE, respectively, and this compound produced strong Ca^{2+} signals in cells expressing SERT. In DAT- and NET-expressing cells, compound **5R** blocked >50% of the signal, but because of its low potency it was not possible to test higher concentrations to determine its efficacy. As mentioned above **5R** works as a weak partial substrate and blocks 5-HT-induced Ca^{2+} signals only by 60%.

Correlation between synaptosome and Ca^{2+} assays

It is well known that substrates interact with monoamine transporters to produce release of neurotransmitters concomitant with a substrate-induced ionic current through the transporters. Although both properties of substrates are accepted, the correlation between these two substrate effects is difficult to establish. Here we performed a linear correlation analysis between the potencies measured in the Ca^{2+} assays (i.e., electrophysiological signals) and synaptosome release assays (i.e., efflux of tritiated substrates) described above (Table 2 and Table 3, respectively). Compounds that produced full efficacy in the synaptosome release assay and induced signals in the Ca^{2+} assay were included in this correlation analysis. Compounds **4S** and **4R** that produced partial release on DAT and worked as blockers in the Ca^{2+} assay were included in the inhibitor analysis (described below). In addition, effects of compound **4R** on NET were also excluded from the analysis since the compound was not efficacious for producing Ca^{2+} signals in the Ca^{2+} assay. A total of ten conditions were included in the correlation of releasers that yielded a correlation coefficient (r) of 0.86 and slope of 1.03 ± 0.15 ($p=0.001$, Figure 1 left panel). This analysis shows a high potency cluster composed by **3S** actions at DAT, SERT and NET and **3R** action at NET, showing that **3S** is a potent non-specific releaser, whereas **3R** is a potent preferential releaser at NET. Then in decreasing order of potencies, compound **4S** actions at NET and SERT cluster together, followed by the cluster formed by **5S**, **3R** and **4R** actions at SERT, and the least potent is **3R** action at DAT (Figure 1 left panel).

Then a similar correlation analysis for inhibitors was performed; in this analysis the potencies of compounds to block signals induced by neurotransmitters in the Ca^{2+} assay were correlated with the potencies of compounds to block uptake of neurotransmitters in synaptosomes (data from Table 1 and Table 3, respectively). In this analysis substrates were excluded. The action of compound **5R** at SERT was excluded from the analysis since it was not fully efficacious and showed partial substrate effects in the Ca^{2+} assay. A total of 6 conditions were included in the inhibition correlation assay giving a correlation coefficient (r) of 0.83 and slope of 0.87 ± 0.20 ($p = 0.012$, Figure 1 right panel). The most potent inhibitor of the series was **4S** on DAT, a cluster composed by **5S** effects at SERT and NET appeared next at lower potency, and the weakest cluster was the effect of **4R** at DAT and **5R** at DAT and NET (Figure 1 right panel).

Intracranial self-stimulation

Across the 14 rats used in this study, the mean $\pm$ SEM maximal control rate (MCR) was 57 ± 1.6 stimulations per trial, and the mean $\pm$ SEM for the total baseline stimulations per component was 275 ± 13 . Figure 2 shows effects of each test drug on full ICSS frequency-rate curves, and Figure 3 shows summary data for effects collapsed across all frequencies. Each of the compounds produced significant dose-dependent change in ICSS, and results are reported below for interactions between drug dose and brain-stimulation frequency in the two-way ANOVA of drug effects on full frequency-rate curves in Figure 2. Only the *S* enantiomers of *N*-methyl 4-MA (**3S**) [$F(27,135) = 10.73$, $p < 0.0001$] and *N*-ethyl 4-MA (**4S**) [$F(27,135) = 11.17$, $p < 0.0001$] produced abuse-related ICSS facilitation. Specifically, for *S*(+)-*N*-methyl 4-MA, 0.32 mg/kg produced exclusive ICSS facilitation at intermediate frequencies (1.9–1.95 log Hz), whereas 1.0 mg/kg produced mixed effects that included both ICSS facilitation at intermediate frequencies (1.8–1.95 log Hz) and ICSS depression at high frequencies (2.15–2.2 log Hz), and the highest dose of 3.2 mg/kg produced exclusive ICSS depression across a broad range of high frequencies (2–2.2 log Hz). Relative to the *S*(+)-*N*-methyl analog, *S*(+)-*N*-ethyl 4-MA produced weaker but significant ICSS facilitation at a dose of 0.32 mg/kg and more prominent ICSS depression at higher doses. All the remaining compounds/isomers produced only dose-dependent ICSS depression [*S*(+)-*N*-propyl 4-MA (**5S**), $F(27,135) = 8.753$, $p < 0.0001$; *R*(-)-*N*-methyl 4-MA (**3R**), $F(27,135) = 13.26$, $p < 0.0001$; *R*(-)-*N*-ethyl 4-MA (**4R**), $F(36,180) = 12.08$, $p < 0.0001$; *R*(-)-*N*-propyl 4-MA (**5R**), $F(36,180) = 14.38$, $p < 0.0001$]. Regarding relative potency, the *S* isomers were more potent than their corresponding *R* isomers, and within a stereoisomeric class, the analogs with *N*-methyl and *N*-ethyl substituents were approximately equipotent with each other and more potent than the *N*-propyl analog isomers.

DISCUSSION

In this study we describe the pharmacological effects of enantiomers of *N*-methyl (**3S** and **3R**), *N*-ethyl (**4S** and **4R**), and *N*-propyl (**5S** and **5R**) variants of the *N*-alkyl 4-MA scaffold on monoamine transporters, and we further characterize their abuse-related effects.

The differential classification of test drugs as substrate or blocker at monoamine transporters is a tool that can help to predict the action of drugs in the brain. Substrates permeate into

neurons through the transporters, produce inward currents, and induce neurotransmitter release by reverse transport, whereas blockers cannot permeate cells, and their action is restricted to competition with the endogenous substrate.²⁷ Acutely, substrates can account for higher concentrations of extracellular neurotransmitter than blockers, and substrate-induced inward current are correlated with long-term depletion of neurotransmitters in the brain.²⁸

The classification of test compounds as blockers or substrates can be attained by measuring a set of properties when the compounds interact with monoamine transporters. Common features of blockers are that they compete the uptake of substrates, they produce partial to no release in synaptosomes preloaded with a radioactive substrate, and they do not generate inward current upon interaction with the transporter. Substrates also can compete uptake of other substrates, but they differ from blockers in producing fully efficacious release from preloaded synaptosomes, and they induced inward depolarizing currents in cells expressing transporters.^{15,21,23,29}

In agreement with previous studies with the racemate,¹⁵ **3S** is a potent releaser that showed poor selectivity among transporters in synaptosomes. Similarly, this compound showed high potency in activating Ca^{2+} signals in cells expressing Ca^{2+} channels and every monoamine transporter tested, which is an indirect measure of the drug-induced inward depolarizing ionic current. In addition, both assays agreed that **3R** compared to **3S** retains potency at NET as a releaser, accompanied by a ~1 log unit decrease in potency at DAT and SERT. These data suggest that **3S** is a potent and nonspecific substrate for all three monoamine transporters, whereas **3R** is a substrate that displays modest selectivity for (although not stereoselectivity at) NET. A phenylethylamine closely related in structure to **3** is methamphetamine (i.e., **3** minus the 4-methyl substituent); although methamphetamine displays stereoselectivity at DAT and SERT as a releaser in synaptosomes (*S* > *R*), it too, lacks stereoselectivity at NET and both isomers are potent releasers.²⁹ Subtle differences in the protein structure of NET vs DAT or SERT might account for the inability of NET to discriminate enantiomers arising from a chiral α -carbon in the phenylethylamine chemical structure.

Compound **4** in the racemate form showed partial release (~50% of maximal release) via DAT in synaptosomes, but in the Ca^{2+} assay, this racemate showed clear blocker activity; it did not generate a Ca^{2+} signal, but it blocked dopamine signals in a dose-dependent manner.¹⁵ From these data, it was concluded that compound **4** is a transitional ligand that induces structural DAT conformations that allow slow and submaximal release in the absence of inward current. An alternative hypothesis, not tested in the previous work, is that enantiomers of **4** have opposite effects on DAT to produce this intermediate action, but the present results do not support this hypothesis. Here we show that both **4S** and **4R** are partial releasers at DAT in synaptosomes, showing efficacies of 56 and 51% of the maximal release, respectively. Similarly, each enantiomer showed blocker effects in DAT-expressing cells in the Ca^{2+} assay. Thus, both enantiomers produce similar effects to each other and to the racemate in both assays.

Interestingly, **4S** and **4R** when tested at NET using the Ca^{2+} assay produced Ca^{2+} signals that were not fully efficacious (57% and 35% of the NE signal, respectively), but in both cases these compounds were full releasers in the synaptosome assay, supporting that substrates that generate current are full releasers even if the magnitude of the inward current is partial. Currents through transporters have similar properties to ion channels; moreover, single channel events have been measured in membrane patches expressing monoamine transporters resembling *bona-fide* ion channels.^{30,31} The magnitude of the macroscopic current (whole cell current) is the sum of many individual opening events for individual transporters, and variables such as the open probability of the single event, unitary conductance, and the total number of transporters might account for the total current in the cell. Compounds **4S** and **4R** acting on NET can produce lower open probability or reduced unitary conductance compared to NE, and this could explain their reduced efficacy observed in the Ca^{2+} assay, but this does not appear to interfere with the release potential of these agents in synaptosomes. The action of **4S** compared to **4R** on the three transporters was qualitatively similar, but quantitatively the *S* enantiomer was always more potent than *R*. This stereoselectivity was greatest for DAT blocker activity, but was still noticeable for NET and SERT substrate activity.

The results with compounds **5S** and **5R** in the synaptosome and Ca^{2+} assays were consistent in identifying both enantiomers as blockers at DAT and NET where *S* was the more potent enantiomer. Similarly, **5S** functioned as a full substrate/releaser at SERT in both assays. By contrast, minor differences across assays were noted for **5R** at SERT. The synaptosome release assay showed **5R** as inactive, whereas in the Ca^{2+} assay, this compound partially blocked the 5-HT signal and produced a weak Ca^{2+} signal only at very high concentration (100 μM), suggesting that it is a weak blocker (or weak partial substrate).

Overall, the pharmacological characterization of these agents shows that **3S** is a non-selective substrate with good activity at all three monoamine transporters, and its slow transition to **4S** and then **5S** gradually results in a unique and selective substrate/releaser profile at SERT, accompanied by a stepwise transition from substrate to blocker at DAT and NET, and a decrease in the overall observed potency. Compound **3R**, although a substrate/releaser at all three transporters, displays some selectivity for NET; the transition to **4R** blunts its activity at DAT becoming a weak blocker with partial release activity, decreases potency at NET, and retains full efficacy with similar potency at SERT. Compound **5R** is less active across the board with some partial activity at SERT.

ICSS studies provided an opportunity to evaluate *in vivo* behavioral effects associated with the *in vitro* pharmacological profiles of these *N*-alkyl 4-MA enantiomers. In ICSS, drugs that increase dopaminergic signalling (e.g. amphetamine) often produce leftward shifts in ICSS frequency-rate curves. This effect is often described as “ICSS facilitation,” and because such dopaminergic drugs often have high abuse liability, leftward shifts in ICSS frequency-rate curves, indicative of ICSS facilitation, are often interpreted as an abuse-related effect.³² Conversely, drugs that primarily increase noradrenergic or serotonergic signalling (e.g. nisoxetine or fenfluramine, respectively) often produce rightward and/or downward shifts in ICSS frequency-rate curves, and because these drugs usually have low abuse liability, these rightward/downward shifts are often interpreted as abuse-limiting

effects. Previously established ICSS studies on stereoselectivity of amphetamine and amphetamine-like compounds suggest a difference in isomer potency for compounds that are releasers at monoamine transporters.^{33–35}

The current ICSS studies corroborate and extend our previously published studies on the actions of *N*-alkyl 4-MA racemates **3–5**.¹⁵ In the racemate study, ICSS facilitation was only observed for *N*-methyl > *N*-ethyl 4-MA (i.e., **3** > **4**).¹⁵ In the current study ICSS facilitation was observed with only the *S* isomers of *N*-methyl-> *N*-ethyl-4-MA (**3S** and **4S**, respectively), suggesting that the abuse-mediated effects of both the *N*-methyl and *N*-ethyl analogs are associated primarily with their *S* isomers. Furthermore, the *S* isomers of *N*-methyl and *N*-ethyl 4-MA were 10- and 3-fold more potent at producing significant ICSS effects than their *R* isomers, respectively. Increasing the *N*-alkyl chain length further, racemic *N*-propyl 4-MA produced an exclusive dose-dependent depression of ICSS;¹⁵ similar effects were found with its individual optical isomers in the present study; however, the *R* isomer produced a more robust depression of ICSS as the dose was increased. As previously established with the racemates of these compounds,¹⁵ increased chain length of the amine substituent caused a decrease in both the effectiveness to produce abuse-related ICSS facilitation and in potency to produce any changes in ICSS. In the current study the same relationship is observed i.e., *S*(+)*N*-methyl *S*(+)*N*-ethyl > *S*(+)*N*-*n*-propyl 4-MA, and *R*(–)*N*-methyl *R*(–)*N*-ethyl > *R*(–)*N*-*n*-propyl 4-MA.

In summary, the current study, coupled with our earlier investigations,^{14,15} provides additional evidence that bulk around the terminal amine of phenylalkylamines such as amphetamine or cathinone (e.g. increasing chain length from NH to *N*-Me, to *N*-Et, to *N*-*n*-Pr, or to other bulky substituents as shown previously¹⁴), and their optical isomers, dictate whether they will behave as MAT releasing agents or as MAT reuptake inhibitors. It seems that the bulkier the *N*-substituent, the more likely the agent will act as a reuptake inhibitor at DAT. We have previously found that tertiary amine analogs of synthetic cathinones behave as DAT reuptake inhibitors, but the present and related studies (e.g.¹⁴) show that secondary amine analogs of amphetamine and cathinone can also play this role if their alkyl-length or bulk is increased. *N*-Alkyl substituted phenylalkylamine analogs, and optical isomers thereof, also have a role in MAT selectivity/function and abuse liability as shown here (and before^{14,15}). Further investigation of *N*-alkyl amphetamine derivatives, and their corresponding β -keto analogs (i.e., synthetic cathinone analogs) is required.

Methods

Chemistry

All commercially available reagents and solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used as delivered. Melting points were measured in glass capillary tubes (Thomas-Hoover melting point apparatus) and are uncorrected. ¹H NMR spectra were recorded with a Bruker 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane as an internal standard. Optical rotations were measured using a Jasco DIP-1000 polarimeter. Reactions and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F254

Merck plates. The purity of the tested compounds was established by elemental analysis (Atlantic Microlabs; Norcross, GA); values for C, H, and N were within 0.4% of theory.

R(-)-N-Methyl-1-(p-tolyl)propan-2-amine hydrochloride (3R)—Methylamine (40% in H₂O, 8.2 mL, 105.3 mmol) was added to a solution of **8S** (0.57 g, 2.5 mmol) in DMF (15 mL) at room temperature. After the mixture was stirred at 50 °C for 12 h, another portion of methylamine (4.1 mL, 52.7 mmol) was added, and the resulting mixture was stirred for 12 h at 50 °C. Then, the mixture was diluted with Et₂O (30 mL) and water (10 mL). The aqueous phase was extracted with Et₂O (4 × 10 mL). The combined extracts were dried (Na₂SO₄), and the solvent was evaporated under reduced pressure to yield 0.22 g of a yellow oil. A saturated HCl solution in anhydrous Et₂O (2 mL) was added to a solution of the free base in anhydrous Et₂O (2 mL) and the reaction mixture was allowed to warm to room temperature and stirred overnight. The precipitate was collected by filtration to yield a white solid which upon recrystallization from EtOH/Et₂O afforded 0.25 g (44%) of **3R** as a white solid: mp 198–199 °C; ¹H NMR (DMSO-*d*₆) δ: 1.09 (d, *J* = 6.5 Hz, 3H, CH₃), 2.29 (s, 3H, CH₃), 2.55 (t, *J* = 5.5 Hz, 3H, CH₃), 2.61 (dd, *J* = 12.3, 13.1 Hz, 1H, CH), 3.16 (dd, *J* = 3.8, 13.1 Hz, 1H, CH), 3.24–3.37 (m, 1H, CHNH), 7.11–7.19 (m, 4H, Ar-H), 9.08 (br s, 2H, NH₂⁺); α_D²⁰ = –6.0° (*c* = 0.5 MeOH); Anal. Calcd for (C₁₁H₁₇N·HCl·0.2 H₂O) C, 64.98; H, 9.12; N, 6.98. Found: C, 65.01; H, 9.15; N, 7.13.

S(+)-N-Methyl-1-(p-tolyl)propan-2-amine hydrochloride (3S)—The compound was synthesized from **8R** using the procedure described above for the synthesis of the opposite isomer. The product (32%) was obtained as a white solid: mp 197–198 °C; ¹H NMR (DMSO-*d*₆) δ: 1.09 (d, *J* = 6.5 Hz, 3H, CH₃), 2.29 (s, 3H, CH₃), 2.55 (t, *J* = 5.5 Hz, 3H, CH₃), 2.61 (dd, *J* = 12.3, 13.1 Hz, 1H, CH), 3.16 (dd, *J* = 3.8, 13.1 Hz, 1H, CH), 3.24–3.37 (m, 1H, CHNH), 7.11–7.19 (m, 4H, Ar-H), 9.08 (br s, 2H, NH₂⁺); α_D²⁰ = +7.0° (*c* = 0.5 MeOH); Anal. Calcd for (C₁₁H₁₇N·HCl) C, 66.15; H, 9.08; N, 7.01. Found: C, 66.40; H, 9.11; N, 6.98.

R(-)-N-Ethyl-1-(p-tolyl)propan-2-amine hydrochloride (4R)—Ethylamine (70% H₂O, 7.0 g, 10.0 mL, 152.8 mmol) was added to a solution of **8S** (1.5 g, 6.6 mmol) in DMF (40 mL) at room temperature. After the mixture was stirred at 50 °C for 12 h, another portion of ethylamine (2.80 g, 4.0 mL, 61.1 mmol) was added, and the resulting mixture was stirred for 12 h at 50 °C. Then, the mixture was diluted with Et₂O (100 mL) and water (30 mL). The aqueous phase was extracted with Et₂O (4 × 10 mL). The combined extracts were dried (Na₂SO₄), and the solvent was evaporated under reduced pressure to yield 0.6 g of a yellow oil. A saturated HCl solution in anhydrous Et₂O (5 mL) was added to a solution of the free base in anhydrous Et₂O (5 mL) and the reaction mixture was allowed to warm to room temperature and stirred overnight. The precipitate was collected by filtration to yield a white solid which upon recrystallization from EtOH/Et₂O afforded 0.24 g (18%) of **4R** as a white solid: mp 204–205 °C; ¹H NMR (DMSO-*d*₆) δ: 1.09 (d, *J* = 6.5 Hz, 3H, CH₃), 1.25 (t, *J* = 7.2 Hz, 2H, NCH₂CH₃), 2.29 (s, 3H, CH₃), 2.59 (dd, *J* = 10.5, 13.1 Hz, 1H, CH), 2.88–3.08 (m, 2H, NCH₂CH₃), 3.20 (dd, *J* = 3.6, 13.1 Hz, 1H, CH), 3.29–3.42 (m, 1H, CHNH), 7.09–7.19 (m, 4H, Ar-H), 9.04 (br s, 2H, NH₂⁺); α_D²⁰ = –3.4° (*c* = 1.0 MeOH); Anal. Calcd for (C₁₂H₁₉N·HCl) C, 67.43; H, 9.43; N, 6.55. Found: C, 67.31; H, 9.49; N, 6.46.

S(+)-N-Ethyl-1-(p-tolyl)propan-2-amine hydrochloride (4S)—The compound was synthesized from **8R** using the procedure described above for the synthesis of the opposite isomer. The product (32%) was obtained as a white solid: mp 203–204 °C; ^1H NMR (DMSO- d_6) δ : 1.09 (d, J = 6.5 Hz, 3H, CH_3), 1.25 (t, J = 7.2 Hz, 2H, NCH_2CH_3), 2.29 (s, 3H, CH_3), 2.59 (dd, J = 10.5, 13.1 Hz, 1H, CH), 2.88–3.08 (m, 2H, NCH_2CH_3), 3.20 (dd, J = 3.6, 13.1 Hz, 1H, CH), 3.29–3.42 (m, 1H, CHNH), 7.09–7.19 (m, 4H, Ar-H), 9.04 (br s, 2H, NH_2^+); α^{D}_{20} = +3.2 (c = 1.3 MeOH); Anal. Calcd for ($\text{C}_{13}\text{H}_{22}\text{N.HCl}$) C, 67.43; H, 9.43; N, 6.55. Found: C, 67.33; H, 9.58; N, 6.41.

R(–)-N-(1-(p-Tolyl)propan-2-yl)propan-1-amine hydrochloride (5R)—Propylamine (7.18 g 10.0 mL, 121.6 mmol) was added to a solution of **8S** (1.5 g, 6.6 mmol) in DMF (40 mL) at room temperature. After the mixture was stirred at 50 °C for 12 h, another portion of propylamine (2.87 g, 4.0 mL, 48.6 mmol) was added, and the resulting mixture was stirred for 12 h at 50 °C. Then, the mixture was diluted with Et_2O (100 mL) and water (30 mL). The aqueous phase was extracted with Et_2O (4×10 mL). The combined extracts were dried (Na_2SO_4), and the solvent was evaporated under reduced pressure to yield 0.6 g of a yellow oil. A saturated HCl solution in anhydrous Et_2O (5 mL) was added to a solution of the free base in anhydrous Et_2O (5 mL) and the reaction mixture was allowed to warm to room temperature and stirred overnight. The precipitate was collected by filtration to yield a white solid which upon recrystallization from $\text{EtOH}/\text{Et}_2\text{O}$ afforded 0.32 g (22%) of **5R** as a white solid: mp 207–208 °C; ^1H NMR (DMSO- d_6) δ : 0.94 (t, J = 7.4 Hz, 3H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.09 (d, J = 6.5 Hz, 3H, CH_3), 1.59–1.74 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.29 (s, 3H, CH_3), 2.57 (dd, J = 10.5, 13.0 Hz, 1H, CH), 2.82–3.07 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 3.18 (dd, J = 3.5, 13.0 Hz, 1H, CH), 3.27–3.44 (m, 1H, CHNH), 7.09–7.21 (m, 4H, Ar-H), 9.20 (br s, 2H, NH_2^+); α^{D}_{20} = –7.2 (c = 1.0 g MeOH); Anal. Calcd for ($\text{C}_{13}\text{H}_{22}\text{N.HCl}$) C, 68.55; H, 9.74; N, 6.15. Found: C, 68.63; H, 9.83; N, 6.04.

S(+)-N-(1-(p-Tolyl)propan-2-yl)propan-1-amine hydrochloride (5S)—The compound was synthesized from **8R** using the procedure described above for the synthesis of the opposite isomer. The product (22%) was obtained as a white solid: mp 208–209 °C; ^1H NMR (DMSO- d_6) δ : 0.94 (t, J = 7.4 Hz, 3H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.09 (d, J = 6.5 Hz, 3H, CH_3), 1.59–1.74 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.29 (s, 3H, CH_3), 2.57 (dd, J = 10.5, 13.0 Hz, 1H, CH), 2.82–3.07 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 3.18 (dd, J = 3.5, 13.0 Hz, 1H, CH), 3.27–3.44 (m, 1H, CHNH), 7.09–7.21 (m, 4H, Ar-H), 9.20 (br s, 2H, NH_2^+); α^{D}_{20} = +7.5 (c = 0.6 MeOH); Anal. Calcd for ($\text{C}_{13}\text{H}_{22}\text{N.HCl}$) C, 68.55; H, 9.74; N, 6.15. Found: C, 68.58; H, 9.90; N, 6.11.

S(+)-1-(p-Tolyl)propan-2-ol (7S)—In a 2 neck flask, CuI (1.14 g, 6.0 mmol) was added to a 1M solution of *p*-tolylmagnesium bromide solution (5.86 g, 30.0 mmol) in anhydrous THF (30 mL) under a N_2 atmosphere and cooled to –35 °C. The reaction mixture was allowed to stir for about 30 min, then a solution of *S*(+)-propylene oxide (1.74 g, 15.0 mmol) in 16 mL of anhydrous THF was added slowly with a syringe. Stirring was continued 2 h at –35 °C and for 2 h at room temperature. The reaction mixture was quenched with aqueous ammonium chloride solution. The aqueous phase was extracted with EtOAc (3×40 mL). The combined organic portions were washed with saturated aqueous NaCl solution, dried

(MgSO₄), and evaporated under reduced pressure to give a black oil. The crude compound was purified using flash chromatography (hexane/EtOAc, 80/20) to afford 4.1 g of **7S** (92%) as a colorless oil. ¹H NMR (CDCl₃) δ: 1.16 (d, *J* = 6.1 Hz, 3H, CH₃), 2.25 (s, 3H, CH₃), 2.57 (dd, *J* = 8.0, 13.5 Hz, 1H, CH), 2.67 (dd, *J* = 4.8, 13.5 Hz, 1H, CH), 3.85–3.90 (m, 1H, CH), 6.99–7.08 (m, 4H, Ar-H); α_D₂₀ = +11.8 (*c* = 1.5 MeOH).

R(-)-1-(p-Tolyl)propan-2-ol (7R)—The compound was synthesized using the procedure described above for the synthesis of the opposite isomer. The product (99%) was obtained as a colorless oil. ¹H NMR (CDCl₃) δ: 1.16 (d, *J* = 6.1 Hz, 3H, CH₃), 2.25 (s, 3H, CH₃), 2.57 (dd, *J* = 8.0, 13.5 Hz, 1H, CH), 2.67 (dd, *J* = 4.8, 13.5 Hz, 1H, CH), 3.85–3.90 (m, 1H, CH), 6.99–7.08 (m, 4H, Ar-H); α_D₂₀ = -12.4 (*c* = 6.5 MeOH).

S(+)-1-(p-Tolyl)propan-2-yl methanesulfonate (8S)—Methanesulfonyl chloride (4.0 g, 40.8 mmol) was added dropwise over 15 min to an ice-cold stirred solution of **4S** (4.0 g, 27.2 mmol) and Et₃N (8.25 g, 81.6 mmol) in anhydrous CH₂Cl₂ (50 mL). The resulting mixture was allowed to warm to room temperature and stirred for 12 h. After the addition of 25 mL of CH₂Cl₂, the solution was washed with water (3 × 30 mL), and brine, dried (Na₂SO₄), and evaporated to dryness under reduced pressure to give the crude mesylated product. The crude compound was purified using flash chromatography (hexanes/EtOAc, 80/20) to afford 4.5 g (72%) of **8S** as colorless crystals. mp = 69–70 °C; ¹H NMR (CDCl₃) δ: 1.37 (d, *J* = 6.2 Hz, 3H, CH₃), 2.24 (s, CH₃, CH₃), 2.47 (s, 3H, SO₂CH₃), 2.76 (dd, *J* = 5.4, 13.5 Hz, 1H, CH), 2.87 (dd, *J* = 7.9, 13.5 Hz, 1H, CH), 4.73–4.84 (m, 1H, CH), 6.99–7.08 (m, 4H, Ar-H); α_D₂₀ = +28.0 (*c* = 4.5 CHCl₃).

R(-)-1-(p-Tolyl)propan-2-yl methanesulfonate (8R)—The compound was synthesized from **7R** using the procedure described above for the synthesis of the opposite isomer. The product (77%) was obtained as colorless crystals. mp = 69–70 °C; ¹H NMR (CDCl₃) δ: 1.37 (d, *J* = 6.2 Hz, 3H, CH₃), 2.24 (s, CH₃, CH₃), 2.47 (s, 3H, SO₂CH₃), 2.76 (dd, *J* = 5.4, 13.5 Hz, 1H, CH), 2.87 (dd, *J* = 7.9, 13.5 Hz, 1H, CH), 4.73–4.84 (m, 1H, CH), 6.99–7.08 (m, 4H, Ar-H); α_D₂₀ = -27.0 (*c* = 2.5 CHCl₃).

Transporter uptake and release

Fresh synaptosomes were prepared and used in uptake and release assays following procedures previously described.^{15,36} Briefly, caudate tissue from rat brains was the source of synaptosomes used in DAT assays, whereas the rest of the brain (minus the cerebellum) was used to prepare synaptosomes for SERT and NET assays. The radioactive tracers used in uptake assays were [³H]dopamine, [³H]5-HT, and [³H]norepinephrine to measure DAT, SERT and NET activity, respectively. For the release assay [³H]MPP⁺ was used to preload synaptosomes for DAT and NET assays, and [³H]5-HT was used for SERT assays. Synaptosome assays were terminated by rapid vacuum filtration and retained radioactivity was determined by liquid scintillation. For the release assays 100% release efficacy (%e_{max}) corresponds to the maximal release evoked by the non-specific substrate tyramine at 10 μM concentrations for DAT and NET assays or 100 μM concentrations for SERT assays. Effects of test drugs on uptake inhibition and release were analysed by nonlinear regression using GraphPad Prism 6 as described previously.¹⁵

Intracellular Ca^{2+} flux

Ca^{2+} determinations were performed, as previously described, in cells permanently expressing DAT, NET and SERT and transfected with voltage-gated Ca^{2+} channels.^{15,24,25} Briefly, stable cell lines expressing each transporter were developed using the FlpIn-TREx system (Invitrogen). Cells were plated in 96-well flat-bottom imaging plates, then were co-transfected with plasmids coding α_1 , β_3 , $\alpha_2\gamma$ and EGFP in a ratio (1:0.5:1:0.2, in μg) using Fugene 6 as transfecting reagent. The culturing media was supplemented with doxycycline (1 $\mu\text{g}/\text{mL}$) 3 days before the experiment to induce the expression of the transporters. All experiments were done using the $\text{Ca}_v1.2$ (α_{1C}) calcium channel, and because of the reduced efficacy of compounds **4S** and **4R** in hNET-expressing cells, additional set of experiments were shown using $\text{Ca}_v1.3$ (α_{1D}) that has a lower threshold of voltage activation.

The Ca^{2+} determinations were performed using Fura2, a ratiometric Ca^{2+} sensor; experiments were carried out under constant perfusion at 35 °C (ThermoClamp-1, Automate Scientific) in an Olympus IX70 microscope equipped with 20X 0.8NA oil objective and a fluorescence imaging attachment (Till Photonics). Ratio images (340 nm and 380 nm excitation) were acquired at 3 Hz and recoded for off-line analysis. As previously described, the experiments were done using Imaging Solution (IS) consisting of: 130 NaCl, 4 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 Hepes, 10 glucose (in mM), pH 7.3. Test agents that worked as substrate were subjected to the following protocol: cells were first perfused for 10s with IS and then exposed to a positive control (DA, 5-HT or NE, at 10 μM) for 5 s, after 30 s IS wash, cells were exposed to the test agent at a given concentration for 5 s and then to a final IS wash for additional 30 s. For blockers: cells were perfused for 10 s with IS, then exposed to a positive control (DA, 5-HT or NE, at 10 μM) for 5 s, followed by an IS wash for 30 s, then the agent at a given concentration was perfused for 30 s and then exposed to agent plus positive control for additional 5 s, and then cells underwent a final wash of 30 s. Each well was exposed to a single concentration of the test agent. The analysis of recorded images was performed using Till Photonics off-line analysis software. The fluorescence intensity of individual cells, normally 5 to 10 cells per well, that responded to the positive control are quantified and save as a temporal numeric series. For each dose-response determination 2–5 wells were collected per dose, normally 2 to 3 different sets of experiments were used to collect the data; thus, each concentration point has data of at least 4 wells (> than 20 cells). The amplitude of the signal produce by the test drug (in the case of substrates) or the amplitude of the signal of the endogenous neurotransmitter in the presence of the test drug (in the case of blockers) is divided by the signal produced by the positive control for each individual cell. This ratio was used to generate a dose-response curve using GraphPad Prism 5.0 software as described previously.¹⁵

Correlation between the synaptosome and Ca^{2+} assays

Linear correlation was performed between the results obtained in synaptosomes and Ca^{2+} assays to characterize substrates and blockers of monoamine transporters. To study substrates (or releasers), the EC_{50} values obtained in the synaptosome release assay were correlated with the EC_{50} value measured to generate signals in the Ca^{2+} assay. Each (x,y) coordinate corresponds to the EC_{50} value of a single compound tested in a single monoamine transporter in the Ca^{2+} or synaptosome assay, respectively. To study blockers (or

inhibitors), the IC₅₀ values determined in the synaptosome inhibition assay were correlated with the IC₅₀ values obtained in the Ca²⁺ inhibition assay. For inhibitors each (x,y) coordinate is the IC₅₀ value of a single compound effect on a single transporter extracted from the Ca²⁺ and synaptosome inhibition assay, respectively. The correlation analysis was performed using GraphPad Prism 5.0 software.

Intracranial self-stimulation (ICSS)

Subjects—Studies were conducted using previously described methods⁹ in a total of 14 adult male Sprague-Dawley rats (Envigo, Frederick, MD) that weighed at least 290 g before surgery. Rats had free access to food and water except during test sessions and were housed individually and maintained on a 12-hour light/dark cycle (lights on from 6am to 6pm) in a facility accredited by the Association for the Assessment of Laboratory Animal Care. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.³⁷

Surgery—Rats were anesthetized with isoflurane (3% in oxygen; Webster Veterinary, Phoenix, AZ) until unresponsive to toe-pinch prior to implantation of stainless steel electrodes (Plastics One, Roanoke, VA). The cathode, which was 0.25 mm in diameter and covered with polyamide insulation except at the flattened tip, was stereotactically implanted into the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to the midsagittal suture, and 8.8 mm ventral to the skull). Three screws were placed in the skull, and the anode (0.125 mm diameter, uninsulated) was wrapped around one of the screws to act as a ground. Dental acrylic was used to fasten the electrode to the screws and skull. Ketoprofen (5 mg/kg) was administered as a postoperative analgesic immediately and 24 h post surgery. Animals were allowed to recover for at least 7 days before ICSS training was started.

Apparatus—ICSS experiments were conducted in operant chambers consisting of sound-attenuating boxes containing modular acrylic and metal test chambers (29.2 cm X 30.5 cm X 24.1 cm) (Med Associates, St. Albans, VT). Each chamber was equipped with a response lever (4.5 cm wide, 2.0 cm deep, 3.0 cm above the floor), three stimulus lights (red, yellow and green) centered 7.6 cm above the lever and arranged horizontally, a 2 W house light, and an ICSS stimulator. Bipolar cables routed through a swivel-commutator (Model SL2C, Plastics One) connected the stimulator to the electrode. MED-PC IV computer software was used to control all the operant programming parameters and obtain data (Med Associates).

Training—At the beginning of each behavioral session the house light was illuminated, and responding under a fixed-ratio (FR 1) schedule produced a 0.5-s train of square-wave cathodal pulses (0.1 ms per pulse) delivered via the intracranial electrode. Illumination of the stimulus lights over the lever accompanied brain stimulation. Initial training sessions lasted 60 min, and the stimulation frequency and intensity were set at 126 Hz and 150 μ A, respectively. Stimulation intensity was then manipulated for each rat to a level sufficient to maintain response rates >30 responses/min (100–290 μ A in this study). Subsequently, behavioral sessions consisted of three 10-min components, each consisting of 10 1-min

frequency trials. The frequency of brain stimulation descended across trials in 10 steps from 2.2 log Hz to 1.75 log Hz in 0.05 log increments. Each trial began with a 10-s time out, during which the house light was off, and responding had no scheduled consequences. During the last 5 s of this time out, 5 noncontingent stimulations at the designated frequency were delivered at 1-s intervals. For the remaining 50 s of each trial, the house light was illuminated, and responding produced brain stimulation at the designated frequency under the FR 1 schedule as described above. Training continued until responding occurred primarily during the first three to six frequency trials of each component. ICSS performance was considered stable when frequency-rate curves were not statistically different over three consecutive days of training as indicated by lack of a significant effect of 'day' in a two-way analysis of variance (ANOVA) with day and frequency as the main effect variables (see Data Analysis below). All training was completed within six weeks of surgery.

Drug testing—Test sessions consisted of three consecutive baseline components followed first by a 10-min time out and then by two test components. A single dose of test drug was administered i.p. at the start of the time out. A total of 6 rats were tested for each drug, and the dose order was varied across rats using a Latin-Square design. Tests with different drugs in a single rat were separated by at least 1 week, and experiments with any single drug were completed prior to beginning tests with another drug. Test sessions were conducted on Tuesday and Friday, and three-component training sessions were conducted on all other weekdays.

Data analysis—The primary dependent variable was the reinforcement rate in stimulations per minute during each frequency trial. The first baseline component of each test session was considered to be a "warm-up" component, and data were discarded. The maximum rate observed during any frequency trial of the second and third baseline components were averaged to yield the Maximum Control Rate (MCR) for that rat on that day. The ICSS rate during each baseline and test trial was then normalized to the MCR using the equation: $\%MCR = (\text{reinforcement rate during a frequency trial} / MCR) \times 100$. Test-component data were averaged first within each rat, and then across rats, to yield test frequency-rate curves after each dose of each drug. Results for each drug were analyzed by two-way ANOVA, with brain-stimulation frequency and drug dose as the two factors. A significant ANOVA was followed by a Holm-Sidak *post hoc* test, and the criterion for significance was set at $p < 0.05$. As an additional summary measure of ICSS, the total number of stimulations was collapsed across all 10 frequency trials for each baseline and test component of each test session. Test data were normalized to baseline data using the equation $\% \text{ baseline total stimulations per component} = (\text{mean total stimulations per test component} / \text{mean total stimulations per baseline component}) \times 100$. Data were then averaged across rats in each experimental condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Two-dimensional visualization of potencies of compounds obtained in synaptosomes and Ca^{2+} assays. The monoamine transporter tested is indicated as N, S, and D for NET, SERT and DAT, respectively. *Left panel*, compound producing full release in the synaptosome assay and producing signals in the Ca^{2+} assay were included in the analysis. The correlation analysis was performed between the EC_{50} to produce release in the synaptosome assay and the EC_{50} to produce signals in the Ca assay. *Right panel*, compounds that produced partial or no release in the synaptosome assay and that do not produce signals in the Ca^{2+} assay were included in the analysis. The correlation analysis was done between the IC_{50} to block neurotransmitter's uptake in the synaptosome assay and the IC_{50} to block the signal induced by neurotransmitters in the Ca^{2+} assay.

Figure 2.

Effects of N-alkyl-4-MA isomers on ICSS frequency-rate curves. Abscissae: frequency of electrical brain stimulation in log Hz. Ordinates: reinforcement rate expressed as percentage of maximum control reinforcement rate (% MCR). Drug doses are indicated in units of mg kg⁻¹. Filled symbols represent frequencies at which ICSS rates after drug administration were statistically different from rates after saline administration as determined by two-way ANOVA followed by Holm-Sidak *post hoc* test, $p < 0.05$. All data are mean $\pm$ SEM for 6 rats.

Figure 3.

Summary of ICSS data for drug effects collapsed across all frequencies. Abscissae: drug dose in mg kg^{-1} . Ordinates: percentage of baseline number of stimulations per component delivered across all brain stimulation frequencies. Points indicate ICSS rate increasing/decreasing effects, relative to Sal (saline). All data show mean $\pm$ SEM for 6 rats.

Scheme 1.

Reagents and conditions: (a) *S*(-)-propylene oxide or *R*(+)-propylene oxide, CuI, THF, N₂, -35 °C, 2 h; (b) MsCl, Et₃N, CH₂Cl₂, 0 °C to rt, 12 h; (c) (i) NH₂R, DMF, 50 °C, 24 h, (ii) HCl/Et₂O.

Table 1Effects of *N*-alkyl 4-MA isomers on uptake inhibition in rat brain synaptosomes.^a

Ligand	DAT IC₅₀ nM (±SEM)	NET IC₅₀ nM (±SEM)	SERT IC₅₀ nM (±SEM)
(±)N-methyl (3)	910	438	171
<i>S</i> (+)N-methyl (3S)	506 ± 33	298 ± 30	102 ± 13
<i>R</i> (-)N-methyl (3R)	5028 ± 8821	431 ± 53	839 ± 84
(±)N-ethyl (4)	795	685	166
<i>S</i> (+)N-ethyl (4S)	346 ± 17	286 ± 18	243 ± 16
<i>R</i> (-)N-ethyl (4R)	4156 ± 213	1970 ± 163	1490 ± 111
(±)N-propyl (5)	3064	2916	984
<i>S</i> (+)N-propyl (5S)	1356 ± 253	1906 ± 306	868 ± 139
<i>R</i> (-)N-propyl (5R)	6181 ± 901	4790 ± 675	3847 ± 166

^aData for the racemates were previously published¹⁵ and are included here only for comparison. All data are expressed as mean ± SD for n = 3 experiments performed in triplicate.

Table 2Effects of *N*-alkyl 4-MA isomers on transporter-mediated release in rat brain synaptosomes.^a

Ligand	DAT EC ₅₀ nM (±SEM) [e _{max} %] ^b	NET EC ₅₀ nM (±SEM) [e _{max} %]	SERT EC ₅₀ nM (±SEM) [e _{max} %]
(±)N-methyl (3)	41	67	67
<i>S</i> (+)N-methyl (3S)	45 ± 6 [104%]	25 ± 3 [107%]	22 ± 3 [104%]
<i>R</i> (-)N-methyl (3R)	476 ± 78 [96%]	45 ± 7 [101%]	175 ± 19 [108%]
(±)N-ethyl (4)	550	182	102
<i>S</i> (+)N-ethyl (4S)	152 ± 28 [56%]	61 ± 10 [103%]	43 ± 4 [100%]
<i>R</i> (-)N-ethyl (4R)	3541 ± 805 [51%]	1190 ± 161 [94%]	327 ± 34 [94%]
(±)N-propyl (5)	Inactive	742	650
<i>S</i> (+)N-propyl (5S)	Inactive	Inactive	197 ± 32 [109%]
<i>R</i> (-)N-propyl (5R)	Inactive	Inactive	Inactive

^aData for the racemates were previously published¹⁵ and are included here only for comparison.^be_{max} is defined as the maximal release induced by 10 μM tyramine for DAT and NET assays and 100 μM tyramine for SERT assays. All data are expressed as mean ± SD for n = 3 experiments performed in triplicate.

Table 3Potency of 4-MA compounds to induce (EC₅₀) or block (IC₅₀; shaded) Ca²⁺ signals.^a

Ligand	DAT EC ₅₀ or IC ₅₀ nM (±SEM)	NET EC ₅₀ or IC ₅₀ nM (±SEM)	SERT EC ₅₀ or IC ₅₀ nM (±SEM)
(±)N-methyl (3)	207	252	479
<i>S</i> (+)N-methyl (3S)	251 ± 4	192 ± 14	273 ± 26
<i>R</i> (-)N-methyl (3R)	3199 ± 1193	190 ± 14	1429 ± 80
(±)N-ethyl (4)	4353	728	683
<i>S</i> (+)N-ethyl (4S)	2785 ± 1289	565 ± 78 ^b	571 ± 77
<i>R</i> (-)N-ethyl (4R)	44490 ± 12990	1168 ± 212 ^b	1615 ± 140
(±)N-propyl (5)	18260	14350	2444
<i>S</i> (+)N-propyl (5S)	4641 ± 756	6662 ± 1050	872 ± 118
<i>R</i> (-)N-propyl (5R)	25040 ± 2071	26000 ± 3514	4280 ± 1338 ^c

^aData on racemates previously published¹⁵ and are included here only for comparison.^bIC₅₀ values obtained using CaV1.3.^cCompound **5R** showed partial substrate effect at SERT; shown here is the IC₅₀ value to block 5-HT-induced Ca²⁺ signals.

Table 4Efficacy of 4-MA compounds to produce or block (shaded) Ca²⁺ signals.

Ligand	DAT % (±SEM)	NET % (±SEM)	SERT % (±SEM)
<i>S</i> (+)-N-methyl (3S)	109 ± 5	102 ± 2	94 ± 2.9
<i>R</i> (-)-N-methyl (3R)	98 ± 14	90 ± 2	107 ± 7.6
<i>S</i> (+)-N-ethyl (4S)	92 ± 2	57 ± 3	91 ± 4
<i>R</i> (-)-N-ethyl (4R)	~100	~35	96 ± 3
<i>S</i> (+)-N-propyl (5S)	~100	~100	83 ± 3
<i>R</i> (-)-N-propyl (5R)	>75	>50	60 ± 7