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Structure-activity relationships of substituted cathinones, with transporter binding, uptake and
release

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Running title page

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HEK, human embryonic kidney; DA, dopamine; 5-HT, 5-hydroxy-tryptamine, serotonin; NE, norepinephrine; hDAT, human dopamine transporter; hSERT, human serotonin transporter; hNET, human norepinephrine transporter; DMEM, Dulbecco's Modified Eagle's Medium; RTI-55, methyl (1*R*,2*S*,3*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; METH, (+)methamphetamine; MDMA, 3,4-methylenedioxymethylamphetamine; MCAT, methcathinone; 3-F MCAT, 3-fluoromethcathinone; 4-Br MCAT, 4-bromomethcathinone; 4-Cl MCAT, 4-chloromethcathinone; 4-FMC, 4-fluoromethcathinone; MDAI, 5,6-methylenedioxy-2-

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aminoindane; 4-MEC, 4-methylethcathinone; 5-MeO-methylone, 5-methoxy-methylone (2-A1MP); ethylone, 3,4-methylenedioxy-N-ethylcathinone (bk-MDEA); 4-FA, 4-fluoroamphetamine; α -PPP, α -pyrrolidinopropiophenone; α -PBP, α -pyrrolidinobutiophenone; α -PVP, α -pyrrolidinovalerophenone; α -PHP, α -pyrrolidinohexiophenone (PV-7); PV-8, 1-phenyl-2-(1-pyrrolidinyl)-1-heptanone; α -PVT, α -pyrrolidinopentiothiophenone; 3,4-MDPPP, 3,4-methylenedioxy- α -pyrrolidinopropiophenone; 3,4-MDPBP 3,4-methylenedioxy- α -pyrrolidinobutiophenone; 4-MeO- α -PVP, 4-methoxy- α -pyrrolidinovalerophenone; 4-MePPP, 4-methyl- α -pyrrolidinopropiophenone; pentedrone, α -methylaminovalerophenone; pentylone, methylenedioxypentedrone; MDPV, 3,4-methylenedioxypyrovalerone; naphyrone, naphpyrovalerone; butylone, β -keto-methylbenzodioxolylbutanamine; Mephedrone, 4-methyl-N-methylcathinone; Methylone, 3,4-methylenedioxy-N-methylcathinone; PCA, p-chloroamphetamine; ICSS, intracranial self-stimulation

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Abstract

Synthetic cathinones are components of “bath salts” and have physical and psychological side effects including hypertension, paranoia, and hallucinations. Herein we report interactions of 20 “bath salt” components with human dopamine, serotonin, and norepinephrine transporters (hDAT, hSERT, and hNET, respectively) heterologously expressed in HEK 293 cells. Transporter inhibitors had nanomolar to micromolar affinities (K_i values) at radioligand binding sites, with relative affinities of hDAT>hNET>hSERT for α -PPP, α -PBP, α -PHP, PV-8, 3,4-MDPPP, 3,4-MDPBP, 4-MePPP, α -PVP, 4-MeO- α -PVP, α -PVT and pentedrone, and hDAT>hSERT>hNET for pentylone. Increasing the α -carbon chain length increased the affinity and potency of the α -pyrrolidinophenones. Uptake inhibitors had relative potencies of hDAT>hNET>hSERT except α -PPP and α -PVT, which had highest potencies at hNET. They did not induce [3 H]neurotransmitter release. Substrates can enter presynaptic neurons via transporters, and the substrates methamphetamine (METH) and 3,4-methylenedioxymethylamphetamine (MDMA) are neurotoxic. We determined that 3-fluoro-, 4-bromo-, 4-chloro-methcathinone and 4-fluoroamphetamine were substrates at all three transporters; MDAI and 4-MEC were substrates primarily at hSERT and hNET, while ethylone and 5-methoxy-methylone were substrates only at hSERT and induced [3 H]neurotransmitter release. Significant correlations between potencies for inhibition of uptake and for inducing release were observed for these and additional substrates. The excellent correlation of efficacy at stimulating release versus K_i/IC_{50} ratios suggested thresholds of binding/uptake ratios above which compounds were likely to be substrates. Based on their potencies at hDAT, most of these compounds have potential for abuse and addiction. 4-Bromomethcathinone, 4-MEC, 5-methoxy-methylone, ethylone and MDAI, which have higher potency at hSERT than hDAT, may have empathogen psychoactivity.

Introduction

New psychoactive substances, including substituted cathinones, are public health and regulatory challenges (Baumann and Volkow, 2016; Griffiths et al., 2013; Spiller et al., 2011). Some compounds, marketed as “bath salts” or “plant food,” have similar activity as the stimulants METH and cocaine or the empathogen MDMA and circumvent legal restrictions of the latter compounds (Iversen et al., 2013). Adverse physical and neuropsychiatric sequelae include tachycardia, brain swelling, hallucinations and paranoia and can result in death (Miotto, et al. 2013; Prosser and Nelson, 2012; Sykutera et al., 2015). Herein we report the pharmacology of second-generation substituted cathinones, and two compounds that target monoamine transporters, 5,6-methylenedioxy-2-aminoindane (MDAI) and 4-fluoroamphetamine (4-FA). Substituted cathinones, structurally related to METH and MDMA, have a β -position ketone as do methcathinone (MCAT) (Fig 1) and (-)-cathinone, the active ingredient in the psychoactive plant product, khat (Glennon, et al., 1987; Kelly, 2011). At least ten substituted cathinones are schedule 1 substances, having no therapeutic uses and high potential for abuse (Drug Enforcement Administration, 2016).

The biochemical pharmacology and behavioral effects of first-generation substituted cathinones have been reported. These include butylone, 4-F MCAT (flephedrone), mephedrone, MCAT, 3,4-methylenedioxypyrovalerone (MDPV), naphyrone, and pyrovalerone, (Baumann et al., 2012; Cameron, et al. 2013; Eshleman et al., 2013; Gatch et al., 2013; Hadlock et al., 2011; Iversen et al., 2013; Lopez-Arnau et al., 2012; Simmler et al., 2014a) and results are summarized in German et al., (2014). Their mechanisms of action are similar, with the compounds primarily binding, with varying affinities and selectivities, to DAT, SERT and NET. Some compounds are transporter blockers, similar to cocaine, while others are substrates, similar to METH. They generally have lower affinities for neurotransmitter receptors than for transporters.

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Second-generation substituted cathinones are synthesized and available on the internet. 3-Fluoromethcathinone (3-F MCAT) and 4-methylethcathinone (4-MEC) were identified in “plant food” being marketed and used as recreational drugs (Archer, 2009; Caudevilla-Galligo et al., 2013; Odoardi et al., 2016). Methylenedioxypentedrone (pentylone) and 4-methyl- α -pyrrolidinopropiophenone (4-MePPP) were found in samples of “NRG-1” and “NRG-3” available from websites (Brandt et al., 2011) and pentylone, α -pyrrolidinovalerophenone (α -PVP), 4-MEC, MDAI, 3,4-methylenedioxy- α -pyrrolidinobutiophenone (3,4-MDPBP) and α -methyaminovalerophenone (pentedrone) were confirmed in human blood specimens (Elliott and Evans, 2014; Marinetti and Antonides, 2013).

Behavioral assays indicate abuse potential of these compounds. 4-MEC, pentedrone, pentylone, 4-MePPP, 3-F MCAT, α -pyrrolidinobutiophenone (α -PBP), and α -PVP stimulate locomotion and substitute, at varying doses, for discriminative stimulus effects of cocaine and METH (Gatch et al., 2015a; Gatch et al., 2015b; Naylor et al., 2015). 3-F MCAT increases mouse locomotor activity, head weaving and salivation, and decreases rotorod performance (Marusich et al., 2012). MDAI, an indane analog of MDMA first synthesized in 1990, substitutes for MDMA and cocaine in drug discrimination assays and produces conditioned place preference (Gatch et al., 2016; Nichols, et al. 1990; Oberlender and Nichols, 1991). In rats, intraperitoneal injections of 4-chloro-methcathinone (4-Cl MCAT) and 4-bromo-methcathinone (4-Br MCAT) produce increases in nucleus accumbens extracellular dopamine (DA) and serotonin (5-HT) levels (Suyama et al., 2016) but cause minimal effects on intracranial self-stimulation (ICSS), while both 4-MEC and α -PVP affect ICSS similar to METH (Watterson et al., 2014). Rats self-administer α -PVP (Aarde et al., 2015).

In pharmacological assays, 4-MEC, 3-F MCAT, 4-FA, pentylone, pentedrone, 3,4-methylenedioxy-N-ethylcathinone (ethylone), MDAI and 4-Br MCAT inhibit uptake via NET,

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DAT, SERT and at 100 μ M, 3-F MCAT, 4-FA and 4-Br MCAT induce neurotransmitter release via these transporters while MDAI, ethylone, pentylone and 4-MEC induce release mainly via SERT (Iversen et al., 2013; Rickli et al., 2015; Simmler et al., 2013; Simmler et al., 2014a; Simmler et al., 2014b). 3,4-MDPPP, 3,4-MDPBP and α -PVP (O-2387) had similar potencies for inhibition of uptake at DAT and NET and much lower potency at SERT (Meltzer et al., 2006; Rickli et al., 2015). 4-MEC and 4-MePPP have differential effects on DAT and SERT function: both are uptake inhibitors at DAT but inactive at releasing [3 H]MPP $^+$ via DAT from rat synaptosomes, while at SERT 4-MEC inhibits uptake and stimulates release but 4-MePPP is inactive (Saha et al., 2015).

The objective of these experiments was to determine and in some cases confirm interactions with monoamine transporters by substituted cathinones that indicate abuse liability. We investigated structure-activity relationship of ten α -pyrrolidinophenones at hDAT, hSERT and hNET and the effect of ortho- and para-substitutions on the phenyl ring of MCAT. We queried if the relationship between binding affinity and potency for inhibition of uptake predicts whether a compound is a transporter inhibitor or substrate. In addition, the relationship between potencies at inhibition of uptake and stimulation of release was assessed.

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Methods

Drugs and Materials

[¹²⁵I]RTI-55, [³H]DA, [³H]5-HT, and [³H]norepinephrine (NE), were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). Cocaine, METH, MDMA, and MCAT were generously supplied by the NIDA Drug Supply Program (Bethesda, MD). 3-F MCAT, MDAI, 4-MEC, ethylone HCl, 4-MePPP, α-PBP, α-PHP, α-PPP, α-PVP, α-PVT, 3,4-MDPBP, pentedrone, pentylone, and 4-FA were supplied by NIDA (Bethesda, MD). 4-Br MCAT HCl, 4-Cl MCAT HCl, 5-MeO-methylone HCl, 3,4-MDPPP HCl, 4-MeO-PVP HCl, PV-8 HCl and α-PHP HCl were purchased from Cayman Chemical (Ann Arbor, MI). FetalClone and bovine calf serum (BCS) was purchased from HyClone (ThermoFisher, Waltham, MA). Most other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Inhibition of [¹²⁵I]RTI-55 binding to, and [³H]neurotransmitter uptake by, hDAT, hSERT or hNET in Clonal Cells

The methods for characterizing radioligand binding and the functional uptake assays have been described (Eshleman, et al., 1999; Eshleman et al., 2013). Human embryonic kidney (HEK-293) cells stably expressing the recombinant human (h)DAT (HEK-hDAT), hSERT (HEK-hSERT) or hNET (HEK-hNET) were used, with the expression vectors pcDNA1 with pBabepuro for antibiotic resistance (hDAT and hSERT) and pcDNA3 (hNET). Cells were cultured in DMEM supplemented with 5% FetalClone, 5% bovine calf serum, penicillin/streptomycin and 2 µg/ml puromycin (hDAT and hSERT) or 10% FetalClone, penicillin/streptomycin and 300 µg/ml G418. (hNET). For [¹²⁵I]RTI-55 competition binding assays, cells were grown to confluence on 150 mm diameter tissue culture dishes. The medium was removed, and cells were rinsed with calcium- and magnesium-free phosphate-buffered saline (cmf-PBS). Lysis buffer (2 mM HEPES with 1 mM EDTA) was added. After 10 min, cells were scraped from plates and

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centrifuged at 30,000 x g for 20 min. The supernatant was removed, and the pellet was resuspended in 50, 15 or 5 ml (hDAT, hSERT, or hNET, respectively) of 0.32 M sucrose /cell culture plate using a Polytron at setting 7 for 10 sec. The resuspension volume was selected to reflect binding of 10% or less of the total radioactivity.

Binding assay conditions: Each assay tube contained 50 μ l of membrane preparation (about 5-15 μ g of protein), 25 μ l of unknown, compound used to define non-specific binding, or buffer (Krebs-HEPES, pH 7.4; 122 mM NaCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 10 μ M pargyline, 100 μ M tropolone, 0.2% glucose and 0.02% ascorbic acid, buffered with 25 mM HEPES), 25 μ l of [125 I]RTI-55 (40-80 pM final concentration) in a final volume of 250 μ l. Membranes were preincubated with unknowns for 10 min prior to the addition of [125 I]RTI-55. Following a 90 min incubation at 18-20°C, binding was terminated by filtration over Filtermat A filters (Perkin Elmer) using a Tomtec 96-well cell harvester. Filters were washed for six seconds with ice-cold saline. Radioactivity remaining on the filter was determined using a Perkin Elmer μ beta-plate reader. Specific binding was defined as the difference in binding observed in the presence and absence of 5 μ M mazindol (HEK-hDAT and HEK-hNET) or 5 μ M imipramine (HEK-hSERT).

For [3 H]neurotransmitter uptake assays, cells were grown to confluence as described above. The medium was removed, and cells were washed with cmf-PBS. Cells from each plate were gently scraped and then triturated with a pipette in 3 ml Krebs Hepes. Two cell plates provided enough cells for four concentration-response curves.

Uptake inhibition assay conditions: Krebs-HEPES and test compound, compound used to define non-specific uptake, or buffer and cells (50 μ l) were added to vials and placed in a 25°C water bath. Cells were preincubated with unknowns for 10 min prior to the addition of [3 H]dopamine (hDAT), [3 H]serotonin (hSERT), or [3 H]norepinephrine (hNET) (20 nM final concentration) in a

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final volume of 500 μ l. Specific uptake was defined as the difference in uptake observed in the presence and absence of 5 μ M mazindol (HEK-hDAT and HEK-hNET) or 5 μ M imipramine (HEK-hSERT). Filtration using a Tomtec 96-well cell harvester through Filtermat A filters presoaked in 0.05% polyethylenimine was used to terminate uptake after 10 min.

Biogenic amine transporters: [3 H]Neurotransmitter release

The methods for characterizing drug-induced release of pre-loaded [3 H]neurotransmitter from HEK-hDAT, HEK-hSERT and HEK-hNET cells have been described previously (Gatch, et al., 2011; Eshleman, et al., 2013). A 20-channel Suprafusion 2500 device (Brandel, Gaithersburg, MD) was used, with a perfusion speed to yield a flow rate of 0.8 ml / min. For HEK-hDAT and HEK-hSERT assays, a 150 mm diameter plate of cells was scraped into 8 ml Krebs HEPES buffer, centrifuged at 500 rpm for 7 min, and resuspended in 8 ml Krebs HEPES. For HEK-hNET assays, three plates of cells were prepared as above. To load the cells with [3 H]neurotransmitter, uptake was conducted for 15 min at 30°C with 120 nM [3 H]DA (9.9 Ci/mmol, hDAT), 30 min at 25°C with 20 nM [3 H]5-HT (29 Ci/mmol, hSERT) or 15 min at 30°C with 120 nM [3 H]NE (6.5 Ci/mmol, hNET). Cells were centrifuged, resuspended in 7 ml Krebs HEPES, and cell suspension (280 μ l) was added to the superfusion device, using polyethylene discs (Brandel) in the reaction tubes. Release was conducted at 30°C (hDAT) or 25°C (hSERT and hNET). Buffer was perfused for 12 min (hDAT and hSERT) or 15 min (hNET), with the last 6 min (3 x 2 min fractions) collected. The cells were then perfused with drug solutions and 22 min (11 x 2 min fractions) of effluent were collected. To release the remaining radioactivity in the cells, SDS (1%) was perfused, and 4 fractions, 2.5 min each, were collected. Radioactivity in the samples was determined using conventional liquid scintillation spectrometry.

Data analysis

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For competition binding results, data were normalized to the specific binding in the absence of drug. Three or more independent competition experiments were conducted with duplicate determinations. GraphPAD Prism (La Jolla, CA) was used to analyze the ensuing data, with IC_{50} values converted to K_i values using the Cheng-Prusoff (Cheng and Prusoff, 1973) equation ($K_i = IC_{50} / (1 + ([drug^*] / K_d drug^*))$), where drug* was [125 I]RTI-55, and the K_d was determined using the described assay conditions. The K_d values used in the equations are given Eshleman et al., 2013. Differences in affinities were assessed by one way ANOVA using the logarithms of the K_i values for test compounds and standards. Dunnett's multiple comparison test was used to compare test compounds to a drug standard, typically METH.

For release assays, fractional release was defined as the amount of radioactivity in a fraction divided by the total radioactivity remaining in the sample, which was determined by summing the cpms in that fraction and all later fractions. For each time course, the area under the curve (AUC) was calculated using GraphPad Prism with baseline defined as the average of the two lowest fractions of that time course. For each experiment, the AUC of basal release in the absence of drug was subtracted from each AUC before normalizing to the percent of maximal METH-stimulated release (or maximal PCA-stimulated release for some hSERT experiments). For functional assays, GraphPAD Prism was used to calculate either IC_{50} (uptake) or EC_{50} (release) values using sigmoidal dose-response nonlinear regression with data expressed as percent of total specific uptake or percent of maximal METH-stimulated release. Dunnett's multiple comparison test was used to compare test compounds to a drug standard, typically METH. Spearman nonparametric correlation was used to analyze the relationships among affinity for the binding site on the transporters and the potencies for inhibition of uptake and stimulation of release.

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Results

α -Pyrrolidinophenones at hDAT, hSERT and hNET: Inhibition of [125 I]RTI-55 binding and [3 H]neurotransmitter uptake

The affinities of the 18 substituted cathinones, MDAI, 4-FA, METH, MDMA and MCAT and the reference compounds cocaine and mazindol for the hDAT, hSERT and hNET expressed in HEK cells were assessed by inhibition of [125 I]RTI-55 binding (Table 1). The rank order of affinity for the compounds differed among the transporters. For example, the 5 compounds with highest affinity at hDAT and hNET were PV-8, α -PHP, α -PVP, MDPBP and α -PBP, albeit in different rank order, while at hSERT the highest affinity compounds were pentylone, 4-MeO-PVP, 4-Br-MC, 4-MEC, and pentedrone.

The compounds with highest affinities for hDAT and hNET were α -pyrrolidinophenones, which are cathinones with a pyrrolidine group, with varying carbon chain length on the α -carbon. In general, this group of compounds was relatively selective for hDAT. At hDAT, the affinity increased with the length of the carbon chain (K_i in μ M): methyl: α -PPP (1.29), ethyl: α -PBP (0.145), propyl: α -PVP (0.0222), butyl: α -PHP (0.016), pentyl: PV-8 (0.0148) (Table 1). A similar structure-activity relationship was seen when measuring drug potency for inhibition of [3 H]DA uptake. The addition of a 4-methyl group to α -PPP, forming 4-MePPP, had no effect on affinity or potency. The addition of a 4-methoxy group to α -PVP, forming 4-MeO- α -PVP, decreased affinity and potency 6-10 fold. Addition of a 3,4-methylenedioxy moiety to the phenyl ring of α -PPP to form 3,4-MDPPP did not change affinity or potency, while this addition to α -PBP to form 3,4-MDPBP and to α -PVP to form MDPV (Eshleman, et al., 2013) increased affinity but had little effect on potency. The substitution of a thiophene group for the phenyl of α -PVP to form α -PVT decreased affinity 30 fold and potency 15 fold.

The α -pyrrolidinophenones had lower affinity for hNET than for hDAT, and there was a trend for affinity to increase with increasing chain length, with the 3-carbon α -PVP having the highest affinity. Substitutions at the 4 position or addition of a 3,4-methylenedioxy group either had no effect or decreased potency in the [3 H]NE uptake assay.

In general, the α -pyrrolidinophenones had very low affinity for and potency at hSERT (Table 1). As with hDAT, the affinity increased with the length of carbon chain: α -PPP K_i =161.4 μ M to α -PHP K_i =33 μ M (Table 1). Only 4-MeO-PVP had a K_i value below 10 μ M with 10 fold higher affinity than α -PVP. Addition of a 3,4-methylenedioxy group increased affinity 10 (3,4-MDPPP) and 16 (3,4-MDPBP) fold above α -PPP and α -PBP, respectively. This group of compounds also had very low potency at inhibition of [3 H]5-HT uptake. The uptake potency ratio of hDAT to hSERT indicates a very high selectivity for hDAT, ranging from ~2900 for α -PVP to ~18 for 4-MePPP (Table 2). Addition of 4-methyl, 4-MeO- and 3,4-methylenedioxy groups generally increased potency for inhibition of [3 H]5-HT uptake.

Pentedrone is structurally similar to α -PVP, with the pyrrolidine moiety replaced by a secondary nitrogen with a methyl group. Pentedrone has lower affinity and potency at hDAT and hNET, but higher affinity and potency for hSERT, compared to α -PVP. The addition of a 3,4-methylenedioxy group to pentedrone, forming pentylone, had no effect on the affinity at hDAT, but increased affinity and potency at hSERT 5-10 fold and decreased affinity at hNET four fold. Although both compounds had the same order of potency at blocking the transporters (hDAT>hNET>hSERT) as α -PVP, the uptake potency ratios of hDAT to hSERT for pentedrone and pentylone are 51.1 and 4.9, reflecting their increased potency at hSERT (Table 2). For α -PVP, the uptake potency ratio of hDAT to hSERT is 2,893.

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For α -PBP, α -PVP, 4-MePPP, 4-MeO- α -PVP, 3,4-MDPPP, and 3,4-MDPBP the ratio of K_i value to IC_{50} value at each of the transporters is less than six, and for α -PPP, α -PHP, PV-8, α -PVT, pentedrone and pentylone, the ratio is less than six at hDAT and hSERT and less than 13 at hNET (Table 2), indicating that binding affinity is relatively predictive of uptake potency. This is also observed for cocaine and mazindol, which are pure uptake blockers at the transporters. Thus, this pharmacological pattern suggests that these compounds may primarily interact with the transporters as uptake blockers.

Other substituted cathinones, MDAI and 4-FA at hDAT, hSERT and hNET: Inhibition of [125 I]RTI-55 binding and [3 H]neurotransmitter uptake

MCAT had much higher affinity at hDAT and hNET compared to hSERT and a rank order of potency for inhibition of uptake of hNET>hDAT>hSERT. Addition of 3-fluoro, 4-chloro, or 4-bromo substituents to MCAT had minimal effect on affinity or potency at hDAT (Table 1). However, the 4-chloro and 4-bromo moieties increased both affinity and potency at the hSERT, and decreased affinity and potency at the hNET, compared to MCAT. Compared to MCAT, ethylone and 4-MEC had similar affinity but lower potency (6-11 fold) at hDAT, increased affinity (13-25 fold) and potency (44-94 fold) at hSERT, and decreased affinity (4-5 fold) and potency (31-48 fold) at hNET.

In contrast to the α -pyrrolidinophenones, 3-F MCAT, MDAI, 4-MEC, 4-Br MCAT, 4-Cl MCAT, 5-MeO-methylone, ethylone and 4-FA have low affinities for the [125 I]RTI-55 binding site (micromolar range) of hDAT, hSERT and hNET (Table 1). METH, MDMA and MCAT also have low affinities for the [125 I]RTI-55 binding site on the transporters, and these compounds have higher potency at inhibiting uptake at the transporters compared to their K_i values (Tables 1,2). For 3-F MCAT, the ratios of binding K_i to uptake IC_{50} at the 3 transporters were all greater than

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15, and for MDAI the ratio was 11.9 for hDAT and greater than 200 for hSERT and hNET. For 4-MEC, 5-MeO-methylone and ethylone, the ratios for hDAT (3.1, <1.6 and 2.9) and hNET (17, 4, 13.2) were similar to that of the blockers, above, but for hSERT the ratios were much higher. METH and MDMA are substrates at the transporters, and we and others have previously observed that substrates have higher potency at inhibition of uptake compared to affinity for radioligand binding sites (Eshleman et al., 2013; Eshleman et al., 1999; Rothman et al., 2000). Thus, the results suggest that the compounds in this subset are substrates at some or all of the transporters.

hDAT, hSERT and hNET: Substituted cathinone efficacy and potency at stimulating release of preloaded [³H]neurotransmitter.

To determine if the substituted cathinones, MDAI and 4-FA stimulate neurotransmitter release via the transporters, intact cells were preloaded with [³H]neurotransmitter and release assays were conducted with a superfusion device, as described in the methods. This method continually removes released [³H]neurotransmitter from the extracellular buffer and allows release to proceed with minimal or no concurrent reuptake of the released [³H]neurotransmitter. With hDAT, hSERT and hNET cells, release in the presence of cocaine, a transporter blocker, was similar to basal release in the absence of drug indicating that minimal or no released [³H]neurotransmitter was available for reuptake (Fig 2, $p > 0.05$, one way ANOVA followed by Dunnett's multiple comparison test.). In contrast, METH induced robust release via the three transporters, and ethylone induced release only via SERT.

At hDAT, 3-F MCAT (300 nM to 100 μ M) and 4-FA (10 nM-10 μ M), elicited [³H]DA release that peaked about 6 min after addition of drug, with all concentrations above 100 nM having some effect (Fig 3A). 4-Cl MCAT, 4-Br MCAT, 4-FA, and MDAI (Fig 4A, 4B) were partially or fully

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efficacious. In contrast, the α -pyrrolidinophenones, pentedrone, pentylone, 4-MEC, 5-MeO-methylone and ethylone at concentrations ranging from 1 nM to 100 μ M (Fig 4A,4B,5A, and 5B, Table 3) had little to no effect. Comparing the maximal drug-induced releases (Fig 4A and 4B, Table 3, $p < 0.0001$, one-way ANOVA), MDMA, MCAT, 4-Br MCAT, 4-Cl MCAT, and 4-FA elicited maximal [3 H]DA release similar to METH ($p > 0.05$, Dunnett's multiple comparison test), while MDAI and 3-F MCAT were less efficacious ($p < 0.05$). Comparing the potencies of drugs to induce [3 H]DA release (Table 3, $p < 0.0001$, one-way ANOVA), 3-F MCAT had similar potency to METH, while MDAI (55-fold), MDMA (17-fold), MCAT (5.3-fold), 4-FA (3.2 fold), 4-Br MCAT (7.6 fold) and 4-Cl MCAT (6.6 fold) were less potent than METH ($p < 0.05$ -0.001).

At hSERT, 3-F MCAT, 4-Br MCAT, 4-Cl MCAT, 4-FA, MDAI, ethylone, 5-MeO-methylone and 4-MEC elicited release of [3 H]5-HT that peaked about 6 min after addition of drug (Fig 3B,4C,4D, and 5C, Table 3). In contrast, the α -pyrrolidinophenones, pentedrone, and pentylone, had minimal releasing efficacy at concentrations ranging from 1 nM to 100 μ M (Fig 4D,5C and 5D). Compared to the maximal release elicited by METH or PCA (Fig 4C,4D,5C and 5D, Table 3, $p < 0.0001$, one-way ANOVA), MDMA, MCAT, 4-Cl MCAT, MDAI, ethylone, 5-MeO-methylone and 4-MEC elicited similar levels of [3 H]5-HT release ($p > 0.05$, Dunnett's test, Table 3), while 3-F MCAT was less efficacious, and 4-Br MCAT was significantly more efficacious ($p < 0.05$). Compared to the potency of METH (Fig 4C, Table 3, $p < 0.0001$, one-way ANOVA), MDMA (21 fold), 4-Br MCAT (24 fold), 4-Cl MCAT (11 fold), ethylone (15 fold), MDAI (8 fold), 4-MEC (15 fold), and 5-MeO-methylone (6 fold) had higher potencies ($p < 0.01$), while 3-F MCAT and 4-FA had similar potency to METH ($p > 0.05$) and MCAT had lower potency (4.6 fold).

At hNET, 3-F MCAT, 4-MEC, MDAI and 4-FA elicited release of [3 H]NE that peaked 8-16 min after addition of drug (Fig 3C). MCAT, MDAI, 3-F MCAT, 4-FA, 4-Br MCAT and 4-Cl MCAT had similar efficacy compared to METH (Fig 4E,4F,5E and 5F Table 3), while 4-MEC had lower

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efficacy ($p>0.01$) and MDMA had higher efficacy ($p>0.05$). Compared to the potency of METH (Fig 4E, Table 3, $p<0.001$, one-way ANOVA), 4-Br MCAT (12 fold) and 4-Cl MCAT (10 fold) had lower potency and the rest of the substrates had similar potency to METH ($p>0.05$). In contrast, the α -pyrrolidinophenones, pentedrone, pentylone and ethylone had minimal releasing efficacy (Fig 4F, 5E and 5F).

For compounds that stimulated release via the transporters, 3-F MCAT, 4-FA and MCAT had relative potency of hNET>hDAT> hSERT similar to that of METH, MDAI had relative potency of hNET>hSERT>hDAT similar to that of MDMA, and 4-MEC had relative potency of hNET>hSERT>>hDAT. Ethylone and 5-MeO-methylone stimulated release only via hSERT. 4-Br MCAT and 4-Cl MCAT had similar potencies for release at the three transporters. Because only a subset of the drugs had robust effects on release, it is difficult to establish structure-activity relationships across transporters.

As noted above, substrates, defined here as those compounds that elicit non-vesicular release via a transporter, have higher potency in uptake assays compared to their affinity for the [125 I]RTI-55 binding site. We queried whether there is a binding/uptake ratio above which we could predict with some assurance whether a compound is a substrate. Figure 6 shows the relationship between the affinity/potency ratios (listed in Table 2) and the percent maximal release for the 20 compounds reported here, six substituted methcathinones reported earlier (Eshleman, et al., 2013), METH, MCAT, MDMA, S(+)-amphetamine, (+)-fenfluramine, DA, 5-HT and NE at the hDAT, hSERT and hNET. As shown, the relationship was generally hyperbolic, as there was an asymptote for the maximal amount of [3 H]neurotransmitter that could be released. The Spearman's r correlation coefficient was significant for all three transporters. At binding/uptake ratios above approximately 12 for hDAT, all compounds were substrates. For hSERT and hNET, at binding ratios above approximately 10 and 50 (respectively), all

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compounds were substrates, releasing at least 30% of that induced by METH (or PCA for a subset of compounds at hSERT). The highest affinity/potency ratios observed were about 400 for 4-FA at hDAT and hNET and about 200 for MDAI at hSERT. There are 17, 14, and 16 compounds represented in the cluster near the Y axis for hDAT, hSERT and hNET, respectively, that had very low affinity ratios and induced little to no release.

In addition, we wanted to determine if, for substrates, there was a relationship between the potencies to inhibit [3 H]neurotransmitter uptake and to induce [3 H]neurotransmitter release via a given transporter. The relationship between these two values is shown in Figure 7 and there were excellent correlations between these two measures at all three transporters. In general, the EC₅₀ values for release were higher (lower potency) than the IC₅₀ values for uptake. Butylone at hSERT and 4-MEC at hNET were the only compounds that were more potent at inducing release than inhibiting uptake. On average, the EC₅₀ values for release via hDAT were 16.9 ± 4.3 times higher than the uptake IC₅₀ values with a range of 2.7 for (+)fenfluramine to 65 for 4-F MCAT. At hSERT, the EC₅₀ values for release were 7.2 ± 2.2 times higher than the uptake IC₅₀ values with a range of 0.3 for butylone to 36 for (+)fenfluramine. At hNET, the EC₅₀ values for release were 8.2 ± 1.6 times higher than the uptake IC₅₀ values with a range of 0.76 for 4-MEC to 21.6 for MCAT

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Discussion

We have characterized the binding affinities and functional activities of 18 substituted cathinones and two structurally related compounds at the hDAT, hSERT and hNET.

For α -pyrrolidinophenones, increasing the chain length from 1 to 5 carbons (α -PPP to PV-8) increased affinity for hDAT ~100 fold. These results, with increasing affinity and potency at hDAT correlating with increasing carbon chain length off the alpha carbon, are in agreement with Marusich et al. (2014) and Kolanos et al. (2015), who found that the potencies for inhibition at DAT correlate with both the volume of the alpha-substituent and the lipophilic character. We further extended this finding to include hNET, with a ~17 fold increase in affinity from 1 to 3 carbons (α -PVP) and a correlation between affinity at hSERT and alpha-substituent chain length, although all of these compounds had very low affinity for hSERT. Rickli et al., (2015) indicated the same trend for 3,4-methylenedioxy substituted pyrovalerones. The n-pyrrolidino substituent on 4-MePPP is sterically repulsed by bulky side chains in SERT that are found in the orthosteric binding pocket as delineated by the Leu-T structural model, which may account for the low affinity of this entire series for SERT (Saha et al., 2015). The pure transporter inhibitor activity of this group may be due to the n-pyrrolidino substituent, as Sandtner et al (2016) observed that interactions with critical aspartate residues of DAT and SERT are necessary for substrate activity and secondary or tertiary substitution on the nitrogen inhibits interaction with these residues. The high selectivity for hDAT over hSERT would suggest that these compounds may have high abuse liability, consistent with the ability of α -PBP and α -PVP to fully substitute for discriminative stimulus effects of both cocaine and METH and to produce conditioned place preference (Gatch et al., 2015a). In contrast 4-MePPP fully substitutes only for METH, does not produce conditioned place preference (CPP) and in the current study had less selectivity for hDAT, all of which suggests a lower abuse liability as compared to other α -pyrrolidinophenones characterized here. Substrates and inhibitors of the transporters can fully cross-substitute for

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discriminative stimulus effects due to the commonality of increased extracellular neurotransmitter following drug administration (Camp et al., 1994).

Pentedrone and pentylone were two additional transporter inhibitors that had moderate affinity for hDAT. They produce long-lasting locomotor stimulant effects and fully substitute for discriminative stimulus effects of both cocaine and METH and pentedrone increases CPP in mice and is self-administered by rats (Gatch et al., 2015b; Hwang et al., 2015). Both compounds may have abuse potential.

Differing from the α -pyrrolidinophenones, most of the other substituted cathinones were substrates at (some) transporters. Drug substrates for the transporters have several functions, competing with extracellular neurotransmitter for reuptake into the presynaptic neuron and inducing release of intracellular neurotransmitter. There are several theories for the molecular mechanism of substrate-induced release including forward and reverse transport by the same protein, referred to as the “facilitated exchange diffusion model” and forward and reverse transport mediated by different proteins within an oligomeric complex (reviewed in Sitte and Freissmuth, 2015). Another theory is the channel model, whereby substrates are depolarizing and excitatory, causing neurotransmitter release by vesicle fusion (reviewed in De Felice et al., 2014). Certain aspects of this “channel mode” of transporter function are present in our cell expression model. For example, substrates induce inward sodium currents in HEK cells expressing the hNET or hDAT, thereby elevating intracellular sodium to drive reverse transport (Galli et al., 1995; Sitte et al., 1998). However, as HEK cells do not have synaptic vesicles (Hu et al., 2013), release via vesicle fusion is not operational in our model system.

These potentially depolarizing transporter-mediated substrate-induced sodium currents (Sitte et al., 1998; Sonders et al., 1997) cause reverse transport or release of neurotransmitter. The

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exogenous substrate-induced sodium currents can put cells at risk, as (+)fenfluramine is associated with neuronal depletion of 5-HT (Baumann et al., 2014) and amphetamine can indirectly activate voltage-gated Ca^{2+} channels (Cameron et al., 2015). The substrates identified here, especially those that had efficacies similar to METH, may cause depolarization in neurons, as evidenced by the dose-dependent currents elicited by 4-MEC in *Xenopus* oocytes expressing hSERT (Saha et al., 2015).

Diagnostic assays that assess whether a compound is a substrate include electrophysiological measurement of transporter-mediated currents and testing reverse transport under control and intracellular high-sodium conditions (Sitte and Freissmuth, 2015). For example, evidence for substrate versus inhibitor action is the inward depolarizing current elicited by the substrates mephedrone and METH while blockers MDPV and cocaine elicit an outward hyperpolarizing current via DAT expressed in *Xenopus* oocytes (Cameron, et al., 2013). The data in Figure 6 indicate that the binding affinity to uptake inhibition potency ratio is predictive for releasing efficacy, in this model of the transporters expressed in HEK cells with [^{125}I]RTI-55 as the radioligand. For example, the low ratios at hDAT and hNET and much higher ratios at hSERT for 4-MEC, 5-MeO-methylone and ethylone (Table 2) predict that these compounds would be blockers at hDAT and hNET but substrates at hSERT. As anticipated, these compounds were partially to fully efficacious releasers at hSERT (67-99%, Table 3), while none elicited release via hDAT and only 4-MEC minimally released (17%) via hNET. These results agree with Saha et al. (2015), who reported that 4-MEC displays unique activity as a SERT substrate while being a DAT blocker. The binding-to-uptake ratios may be a quick metric to discriminate inhibitors from substrates, as they can be determined more quickly and easily than other methods such as the in vitro efflux methods reported here or the voltage-clamp current measures in *Xenopus* oocytes.

Drugs that are hDAT-selective produce more stimulant and abuse-related effects than substances with mixed action at hDAT and hSERT (Wee et al., 2005; Baumann et al., 2011). The halide substitutions at either the para or ortho position of MCAT decrease the hDAT:hSERT uptake selectivity as MCAT had 136 fold higher potency at hDAT while 3-F MCAT, 4-Cl MCAT and 4-Br MCAT had 60.3, 3.22 and 0.96 fold hDAT selectivity, respectively (Table 2). Similar effects are seen with the hDAT:hSERT release selectivity, with MCAT, 3-F MCAT, 4-Cl MCAT and 4-Br MCAT having 46, 30, 0.68 and 0.29 fold hDAT selectivity, respectively (Table 1 and Table 3). These results are consistent with *para* halide MCAT substitutions decreasing the DAT:SERT release potencies from rat synaptosomes (Bonano et al., 2015). MCAT robustly increases extracellular DA, while 4-F MCAT and 4-Cl MCAT robustly, and 4-Br MCAT modestly, increase both extracellular DA and 5-HT as measured by microdialysis in rats (Suyama et al., 2016). The ratios of (in vivo) potencies to release DA and 5-HT correlate positively with maximal ICSS, a procedure that evaluates abuse potential, and negatively with the steric bulk of the compounds (Suyama et al., 2016). Thus the abuse potential may decrease, while more MDMA-like empathogenic activity may increase, within this series.

With the in vitro model employed here, all substrates, except butylone at hSERT and 4-MEC at hNET, had 7-17 times lower potency on average at inducing release than at inhibition of uptake (Figure 7). This finding may not apply to all model systems. Sandtner et al. (2016) reported higher potencies for release than for uptake for MDMA at the 3 transporters using rat brain synaptosomes whereas MDMA was 3, 3, and 18 fold less potent for release compared to uptake at hNET, hSERT, and hDAT respectively (Tables 2 and 3). The release models are quite different, with HEK cells expressing a single transporter with no blocking agents required, and the superfusion assay involved continuous removal of released substrate from the reaction, compared to rat brain synaptosomes that require blockade of VMAT2 as well as blockade of other transporters, and the “static” release assay does not remove released neurotransmitter

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from the reaction. Another difference between the assays is incubation time, as the release assay with synaptosomes can be up to 30 min, while in the superfusion assay cells are exposed to drug for 22 min, and the peak of release occurs at 6-8 min after drug addition. Both models have similar results discriminating between inhibitors and substrates. The absence of cocaine-induced release at SERT (Fig 2) differs from Scholze et al., (2000), who observed paroxetine-induced “pseudo-release” due to reuptake blockade using attached HEK-hSERT cells with a high concentration of [³H]5-HT and longer washout time.

In summary, except ethylone, MDAI and 5-MeO-methylone, all substituted cathinones and 4-FA had high to moderate potencies for inhibition of uptake at hDAT (less than 1 μM). Compounds with higher potency at hDAT than hSERT, including all the α-pyrrolidinophenones, 3-F MCAT, pentedrone and 4-FA, have a high likelihood of abuse and addiction and similar stimulant properties as METH and/or cocaine. Increasing α-pyrrolidinophenones substituent chain length increases potency at the hDAT, and may increase abuse potential. Compounds with similar potencies at hDAT and hSERT, or higher potency at hSERT than hDAT (4-CI-MCAT, 4-Br MCAT, 4-MEC, 5-MeO-methylone, ethylone, pentylone, and MDAI) may have more empathogenic activity similar to MDMA, but also have some potential for abuse.

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Footnotes

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- b) Portions of this work were presented at the College on Problems of Drug Dependence (2012) annual meeting.
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Legends for Figures

Figure 1. Structures of substituted cathinones, MDMA, METH, 4-FA, and MDAI.

Figure 2. Effect of cocaine, ethylone and METH on [³H]neurotransmitter release. (A) hDAT, (C) hSERT and (E) hNET show the time courses of the average fractional release (mean ± sem) in the presence of buffer, cocaine (10 μM), ethylone (30 μM) or the maximally effective concentrations of METH (10 μM, hDAT; 100-300 μM, hSERT; 1 μM, hNET). (B) hDAT, (D) hSERT and (F) hNET show the average area under the curve (AUC) for fractional release for each condition tested. N=3-6. **, ****, p<0.01, p<0.0001, compared to basal release, one way ANOVA followed by Dunnett's multiple comparison test.

Figure 3. Time courses of [³H]neurotransmitter release induced by the substituted cathinones 3-F MCAT and 4-MEC, the aminoindane MDAI and the substituted amphetamine 4-FA. (A) hDAT, (B) hSERT and (C) hNET cells. Data are representative experiments with a subset of concentrations shown for clarity. Data are normalized to percent release of [³H]neurotransmitter remaining in cells at each time point. The last three buffer fractions prior to addition of drug and the 12 fractions in the presence of drug are shown.

Figure 4. Concentration-response curves of [³H]neurotransmitter release by METH, 3-F MCAT, 4-Cl MCAT, 4-Br MCAT, 4-FA, MDAI, 4-MEC, pentedrone, pentylone, and 5-MeO-methylone. (A,B) hDAT, (C,D) hSERT, and (E,F) hNET cells. The area under the curve (AUC) for each drug concentration was normalized to the maximal effect of METH for that experiment (hDAT, hNET and hSERT) or the maximal effect of PCA (some hSERT). The maximal stimulation of [³H]5-HT release by PCA and METH was similar in experiments when both were tested. Data are the mean and sem of three or more experiments, except when a drug had no releasing efficacy, in which case the data are the mean and range of two experiments.

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Figure 5. Concentration-response curves of [³H]neurotransmitter release by MDMA, MCAT, ethylone α -PPP, α -PBP, α -PVP, α -PHP, PV-8, 4-MePPP, 3,4-MDPPP, 3,4-MDPBP, 4-MeO- α -PVP, and α -PVT. (A,B) hDAT, (C,D) hSERT, and (E,F) hNET cells. The area under the curve (AUC) for each drug concentration was normalized to the maximal effect of METH for that experiment (hDAT, hNET and hSERT) or the maximal effect of PCA (some hSERT). The maximal stimulation of [³H]5-HT by PCA and METH was similar in experiments when both were tested. Data are the mean and sem of three or more experiments, except when a drug had no releasing efficacy, in which case the data are the mean and range of two experiments.

Figure 6. Correlation between the ratio of affinity for the transporter (K_i) and the potency for inhibition of uptake (IC_{50}) to the maximal releasing efficacy of the test compounds. A: hDAT, B: hSERT, C: hNET. The data have been fit to a hyperbolic equation. Values for nonparametric Spearman's correlation (r) and significance are (A) DAT $r=0.84$, $p<0.0001$; (B) SERT $r=0.73$, $p<0.0001$; and (C) NET $r=0.78$, $p<0.0001$. All of the correlations are highly significant. Abbreviation: Amph, (+)-Amphetamine.

Figure 7. Correlation between potencies at inhibition of uptake and stimulation of release. A: hDAT, B: hSERT, C: hNET. The linear regression line is shown. Values for nonparametric Spearman's correlation (r) and significance are (A) DAT $r=0.87$, $p<0.001$; (B) SERT $r=0.75$, $p<0.001$; and (C) NET $r=0.71$, $p<0.006$. All of the correlations are highly significant. Abbreviation: Amph, (+)-Amphetamine.

Table 1. Affinity and potency of substituted cathinones and standard compounds at hDAT, hSERT and hNET.

Drug	Inhibition of [125 I]RTI-55 binding			Inhibition of [3 H]neurotransmitter uptake		
	K _i ± sem (μM) (n)			IC ₅₀ ± sem (μM) (n)		
	hDAT	hSERT	hNET	hDAT [3 H]DA	hSERT [3 H]5-HT	hNET [3 H]NE
α- pyrrolidinophenones						
α-PPP	1.29 ± 0.50 (3)	161.4 ± 6.7 (3)	2.04 ± 0.50 (4)	0.540 ± 0.076 (3)	188 ± 12 (4)	0.305 ± 0.057 (3)
α-PBP	0.145 ± 0.034 (7)	163 ± 41 (5)	0.51 ± 0.13 (5)	0.078 ± 0.011 (3)	67 ± 14 (5)	0.143 ± 0.030 (5)
α-PVP	0.0222 ± 0.0053 (7)	68 ± 18 (3)	0.122 ± 0.029 (3)	0.0197 ± 0.0048 (5)	57 ± 14 (3)	0.046 ± 0.018 (5)
α-PHP	0.0160 ± 0.0044 (8)	33 ± 12 (5)	0.339 ± 0.037 (9)	0.0216 ± 0.0035 (6)	40 ± 11 (3)	0.0363 ± 0.0077 (7)
PV-8	0.0148 ± 0.0041 (5)	39 ± 11 (5)	0.63 ± 0.14 (5)	0.0145 ± 0.0016 (4)	26.8 ± 7.2 (3)	0.0552 ± 0.0054 (4)
4-MePPP	1.12 ± 0.14 (3)	50.01 ± 0.68 (3)	5.87 ± 0.45 (3)	0.566 ± 0.098 (3)	10.34 ± 0.68 (3)	1.07 ± 0.39 (3)
4-MeO-α-PVP	0.317 ± 0.090 (5)	6.07 ± 0.98 (5)	1.12 ± 0.26 (5)	0.1126 ± 0.0094 (3)	4.18 ± 0.28 (3)	0.222 ± 0.054 (3)
3,4-MDPPP	1.10 ± 0.26 (5)	18.7 ± 4.9 (3)	4.9 ± 1.1 (5)	0.409 ± 0.068 (3)	12.8 ± 1.7 (3)	0.881 ± 0.074 (3)
3,4-MDPBP	0.069 ± 0.020 (3)	10.14 ± 0.33 (3)	0.76 ± 0.15 (5)	0.057 ± 0.14 (4)	3.1 ± 1.0 (3)	0.146 ± 0.040 (3)
α-PVT	0.630 ± 0.054 (4)	165.4 ± 5.0 (3)	2.26 ± 0.31 (3)	0.3428 ± 0.0049 (3)	242 ± 41 (4)	0.175 ± 0.038 (4)
Other substituted cathinones						
MCAT	4.78 ± 0.83 (18)	313 ± 46 (14)	3.59 ± 0.52 (21)	0.152 ± 0.015 (18)	20.6 ± 3.6 (20)	0.0297 ± 0.0029 (19)
3-F-MCAT	3.25 ± 0.77 (5)	211 ± 48 (3)	2.92 ± 0.54 (3)	0.214 ± 0.056 (4)	12.9 ± 3.4 (5)	0.0212 ± 0.0010 (3)
4-CI-MCAT	9.41 ± 0.65 (4)	28.7 ± 5.2 (4)	19.6 ± 2.6 (4)	0.208 ± 0.036 (3)	0.67 ± 0.13 (3)	0.0755 ± 0.0072 (3)

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4-Br-MCAT	7.87 ± 0.28 (3)	9.24 ± 0.93 (3)	17.1 ± 1.8 (3)	0.471 ± 0.093 (4)	0.45 ± 0.11 (8)	0.070 ± 0.016 (4)
4-MEC	3.00 ± 0.52 (3)	12.6 ± 2.8 (4)	15.8 ± 3.9 (4)	0.96 ± 0.15 (3)	0.218 ± 0.050 (4)	0.93 ± 0.23 (3)
5-MeO-Methylone	144 ± 54 (4)	27.9 ± 4.2 (3)	76 ± 14 (3)	>92 (4)	1.90 ± 0.11 (4)	19.0 ± 3.8 (4)
Ethylone	5.0 ± 1.6 (3)	23.7 ± 2.1 (4)	18.8 ± 3.0 (3)	1.72 ± 0.45 (3)	0.464 ± 0.075 (4)	1.42 ± 0.20 (3)
Pentedrone	0.339 ± 0.098 (7)	16.4 ± 4.6 (3)	1.95 ± 0.56 (5)	0.1762 ± 0.0089 (3)	9.0 ± 2.3 (3)	0.239 ± 0.067 (3)
Pentylone	0.3942 ± 0.0040 (3)	3.38 ± 0.52 (4)	8.19 ± 0.57 (3)	0.167 ± 0.037 (3)	0.81 ± 0.14 (4)	0.65 ± 0.16 (3)
Non-cathinones						
MDAI	41.3 ± 3.5 (3)	119.1 ± 5.8 (3)	48 ± 11 (4)	3.48 ± 0.46 (3)	0.59 ± 0.13 (4)	0.192 ± 0.036 (3)
4-FA	33.6 ± 3.1 (4)	75.8 ± 6.6 (4)	18.5 ± 4.2 (3)	0.091 ± 0.025 (3)	3.12 ± 0.66 (4)	0.0426 ± 0.0044 (3)
METH	4.58 ± 0.76 (23)	213 ± 29 (20)	1.82 ± 0.23 (19)	0.0667 ± 0.0082 (26)	7.4 ± 1.1 (27)	0.0165 ± 0.0017 (23)
MDMA	32.4 ± 4.9 (15)	23.9 ± 5.5 (14)	26.6 ± 6.4 (16)	0.410 ± 0.045 (13)	0.362 ± 0.053 (14)	0.108 ± 0.015 (13)
Cocaine	0.494 ± 0.037 (29)	0.617 ± 0.045 (27)	1.91 ± 0.23 (25)	0.256 ± 0.022 (26)	0.320 ± 0.034 (33)	0.272 ± 0.024 (23)
Mazindol	0.0296 ± 0.0039 (20)	0.085 ± 0.012 (22)	0.0071 ± 0.0014 (19)	0.0146 ± 0.0016 (21)	0.0435 ± 0.0040 (23)	0.00143 ± 0.00017 (25)

(n) Number of independent experiments conducted in duplicate.

Data are normalized to specific binding or specific uptake in the absence of drugs. Drugs were tested in binding and uptake assays at concentrations ranging from 1 nM to 10 µM, 100 µM or 1 mM. Hill slopes for binding ranged from -0.51 to -1.69.

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Table 2. hDAT uptake selectivity and Ratio of binding affinity to uptake potency of substituted methcathinones and standard compounds at hDAT, hSERT and hNET.

Drug	hDAT uptake Selectivity ^a	Ratio of Ki for inhibition of [¹²⁵ I]RTI-55 binding/ IC ₅₀ for inhibition of [³ H]neurotransmitter uptake		
		hDAT	hSERT	hNET
α- pyrrolidinophenones				
α-PPP	348	2.4	0.9	6.7
α-PBP	859	1.9	2.4	3.6
α-PVP	2893	1.1	1.2	2.7
α-PHP	1852	0.7	0.8	9.3
PV-8	1848	1.0	1.5	11.4
4-MePPP	18.3	2.0	4.8	5.5
4-MeO-α-PVP	37.1	2.8	1.5	5.0
3,4-MDPPP	31.3	2.7	1.5	5.6
3,4 MDPBP	54.4	1.2	3.3	5.2
α-PVT	706	1.8	0.7	12.9
MDPV	110*	1.5*	0.91*	5.7*
Naphyrone	4.7*	0.85*	0.25*	1.7*
Other substituted cathinones				
MCAT	136	31.4	15.2	120.9
3-F MCAT	60.3	15.2	16.4	137.7
4-Cl MCAT	3.22	45.2	42.8	259.6
4-Br MCAT	0.96	16.7	20.5	244.3
4-MEC	0.23	3.1	57.8	17.0
5-MeO-Methylone	<0.02	<1.6	14.7	4.0

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Ethylone	0.27	2.9	51.1	13.2
Pentedrone	51.1	1.9	1.8	8.2
Pentylone	4.9	2.4	4.2	12.6
4-FMC	>36*	38*	ND	276*
Butylone	9.3*	8.2*	5.3*	7.5*
Mephedrone	5.2*	49*	41*	220*
Methylone	5.6*	15*	50*	71*
Non-cathinones				
MDAI	0.17	11.9	201.9	250
4-FA	34.3	369.2	24.3	434.3
METH	111	68.7	28.8	110.3
MDMA	0.88	79.0	66.0	246.3
Cocaine	1.25	1.9	1.9	7.0
Mazindol	3.0	2.0	2.0	5.0

^ahDAT uptake selectivity is calculated as 1/ hDAT IC₅₀ : 1/hSERT IC₅₀

*Data from Eshleman et al., 2013.

ND- Ratio could not be determined.

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Table 3: Potency and efficacy of substituted methcathinones and standard compounds to release preloaded [³H]neurotransmitter from HEK-hDAT, HEK-hSERT and HEK-hNET cells.

Drug-induced release of [³ H]neurotransmitter			
	EC ₅₀ ± sem (μM) (n)		
Drug	% maximum release ± sem*		
α -pyrrolidinophenones			
	hDAT [³ H]DA	hSERT [³ H]5-HT	hNET [³ H]NE
α-PPP	>10 μM (2)	>10 μM (2)	>10 μM (2)
	-0.51 ± 2.06%	-0.58 ± 0.35%	3.7 ± 4.3%
α-PBP	>100 μM (2)	>100 μM (2)	>100 μM (2)
	-1.6 ± 2.8%	1.8 ± 1.6% [#]	-4.1 ± 4.3%
α-PVP	>100 μM (2)	>100 μM (2) [#]	>100 μM (2)
	4.5 ± 1.6%	9.9 ± 1.5%	-1.7 ± 2.9%
α-PHP	>10 μM (2)	>10 μM (2)	>10 μM (2)
	1.47 ± 0.59%	3.2 ± 3.6%	-0.5 ± 1.2%
PV-8	>10 μM (2)	>10 μM (2)	>10 μM (2)
	-2.2 ± 0.73%	-0.53 ± 1.24%	17 ± 11%
4-MePPP	>100 μM (2)	>100 μM (2)	>100 μM (3)
	-1.3 ± 1.7%	-2.1 ± 1.7% [#]	5.2 ± 2.1%
4-MeO-α-PVP	>9.5 (5)	>10 μM (2)	>10 μM (2)
	13 ± 12%	-0.5 ± 1.2%	0.24 ± 0.54%
3,4-MDPPP	>10 μM (2)	>10 μM (2)	>10 μM (2)
	1.34 ± 0.32%	-1.12 ± 0.61%	-0.9 ± 2.7%
3,4-MDPBP	>10 μM (2)	>10 μM (2)	>10 μM (2)
	-0.13 ± 0.24%	0.04 ± 0.54%	10.2 ± 2.4%

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α -PVT	>10 μ M (2) 0.88 \pm 0.02%	>10 μ M (2) 0.43 \pm 0.14%	>10 μ M (2) 4.8 \pm 2.7%
Other substituted cathinones			
MCAT	2.31 \pm 0.76 (11) 85.4 \pm 6.0%	107 \pm 36 (7) 65 \pm 10%	0.64 \pm 0.24 (9) 93.5 \pm 8.2%
3-F MCAT	1.29 \pm 0.42 (6) 63.3 \pm 8.4%	39.0 \pm 4.3 (3) [#] 50.8 \pm 1.4%	0.13 \pm 0.05 (5) 96 \pm 11%
4-Cl MCAT	2.89 \pm 0.99 (3) 83.5 \pm 6.5%	1.98 \pm 0.42 (4) 124 \pm 10%	1.24 \pm 0.44 (6) 84.9 \pm 7.0%
4-Br MCAT	3.3 \pm 1.1 (3) 90 \pm 13%	0.96 \pm 0.13 (4) 131 \pm 13%	1.50 \pm 0.63 (5) 91.8 \pm 6.9%
4-MEC	>100 μ M (3) 0.10 \pm 2.0%	1.52 \pm 0.42 (3) 99 \pm 10%	0.71 \pm 0.20 (5) 17.5 \pm 4.2%
5-MeO-Methylone	>100 μ M (2) 2.1 \pm 0.9%	3.89 \pm 0.41 (3) 67.4 \pm 2.5%	>100 μ M (2) 1.6 \pm 1.3%
Ethylone	>10 μ M (2) 1.22 \pm 0.62%	1.48 \pm 0.25 (4) 86 \pm 15% [#]	>10 μ M (2) 1.3 \pm 1.7%
Pentedrone	>100 μ M (2) 4.2 \pm 1.6%	>100 μ M (2) 7.4 \pm 2.4% [#]	>100 μ M (2) -1.1 \pm 4.4%
Pentylone	>100 μ M (2) 2.4 \pm 1.2%	>100 μ M (2) -5.2 \pm 2.1% [#]	>100 μ M (2) 4.6 \pm 1.5%
Non-cathinones			
MDAI	24 \pm 11 (4) 25.0 \pm 4.9%	2.9 \pm 1.2 (5) 61 \pm 12% [#]	0.57 \pm 0.20 (4) 68 \pm 18%

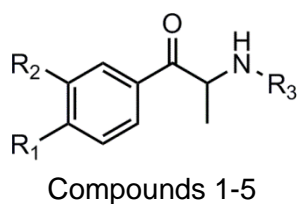
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4-FA	1.40 ± 0.41 (5)	11.1 ± 1.5 (4)	0.144 ± 0.051 (5)
	96 ± 15%	99.5 ± 6.2%	145 ± 25%
METH	0.435 ± 0.074 (13)	23.3 ± 4.2 (10)	0.125 ± 0.024 (8)
	101.3 ± 2.9	93.0 ± 7.5%	102.6 ± 3.0%
MDMA	7.5 ± 2.3 (10)	1.10 ± 0.29 (9)	0.360 ± 0.092 (7)
	94 ± 10%	85.1 ± 7.8%	147 ± 15%
PCA		1.25 ± 0.34 (6)	
		105.5 ± 1.6% [#]	

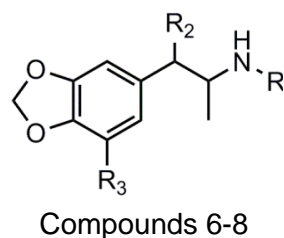
(n) Number of independent experiments.

*Maximum release is defined as the maximum release (maximal AUC) induced by METH (1 or 10 μ M, hDAT; 0.3 or 1 μ M, hNET; 0.3-1 mM, hSERT) or [#]p-chloroamphetamine (PCA, 10 μ M, hSERT) for each experiment. When the data could not be fit by nonlinear regression ($EC_{50} > 10$ or 100 μ M), the maximum release is the average of release at the 3 highest concentrations tested.

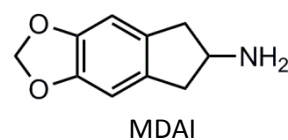
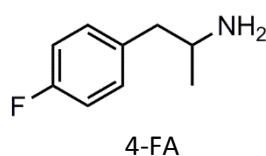
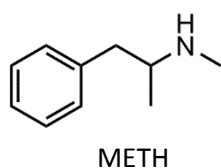
A. Transporter substrates



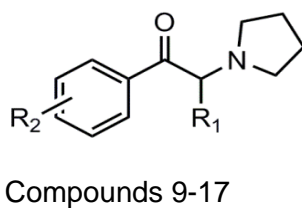
Compounds 1-5	R_1	R_2	R_3
1 MCAT	H	H	CH ₃
2 3-F MCAT	H	F	CH ₃
3 4-Br MCAT	Br	H	CH ₃
4 4-Cl MCAT	Cl	H	CH ₃
5 4-MEC	CH ₃	H	CH ₂ CH ₃



Compounds 6-8	R_1	R_2	R_3
6 MDMA	H	H	H
7 Ethylone	CH ₂ CH ₃	=O	H
8 5-MeO-Methylone	CH ₃	=O	-O-CH ₃



B. Transporter Blockers



Compounds 9-17	R_1	R_2
9 α -PPP	CH ₃	H
10 α -PBP	CH ₂ CH ₃	H
11 α -PVP	(CH ₂) ₂ CH ₃	H
12 α -PHP	(CH ₂) ₃ CH ₃	H
13 PV-8	(CH ₂) ₄ CH ₃	H
14 4-MeO- α -PVP	(CH ₂) ₂ CH ₃	para -O-CH ₃
15 4-MePPP	CH ₃	para -CH ₃
16 3,4 MDPPP	CH ₃	3,4 methylenedioxy
17 3,4 MDPBP	CH ₂ CH ₃	3,4 methylenedioxy

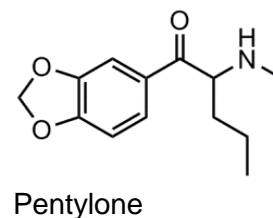
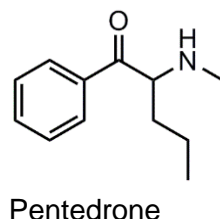
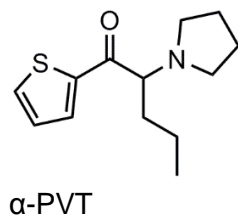


Figure 1

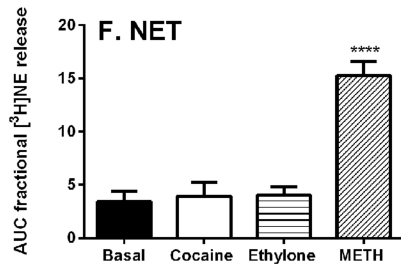
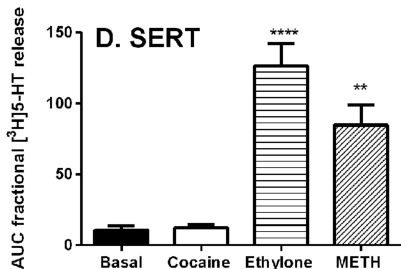
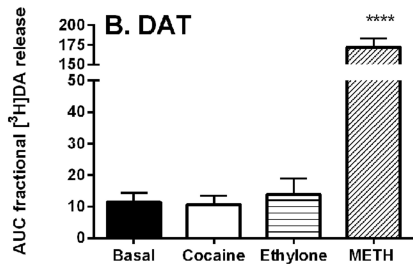
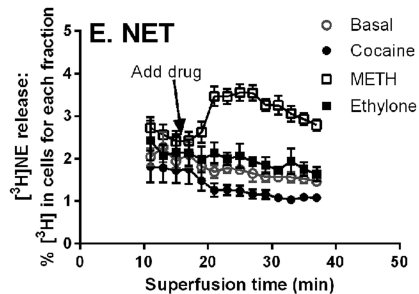
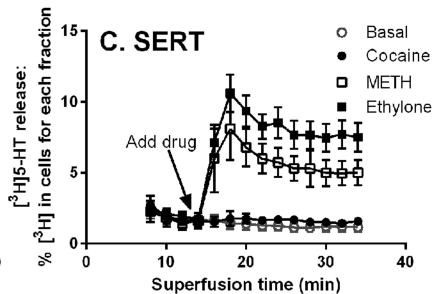
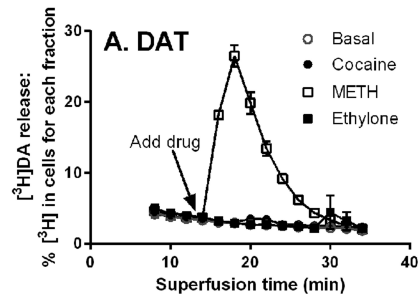
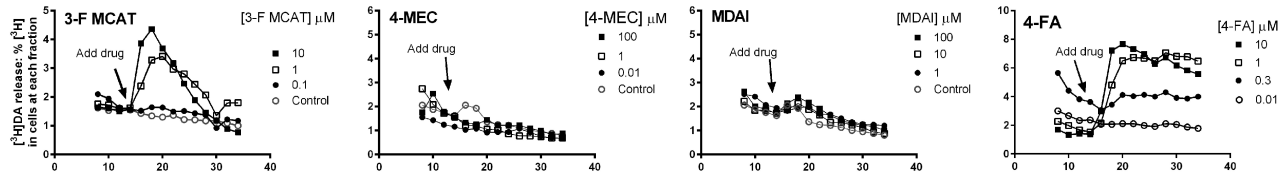
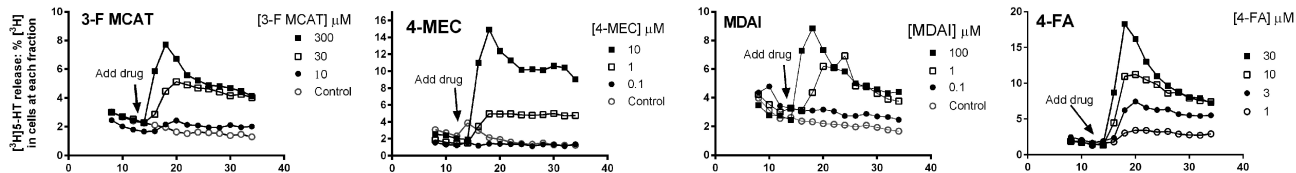


Figure 2

A. hDAT



B. hSERT



C. hNET

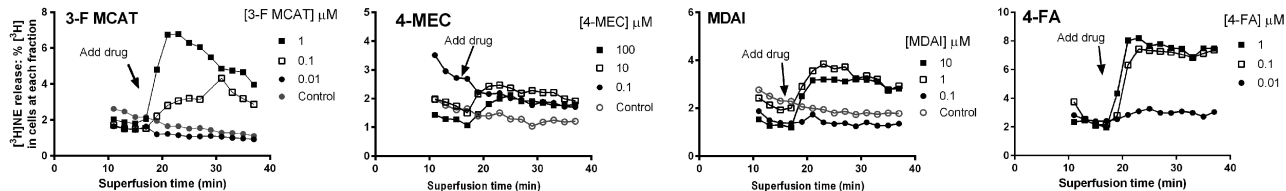


Figure 3

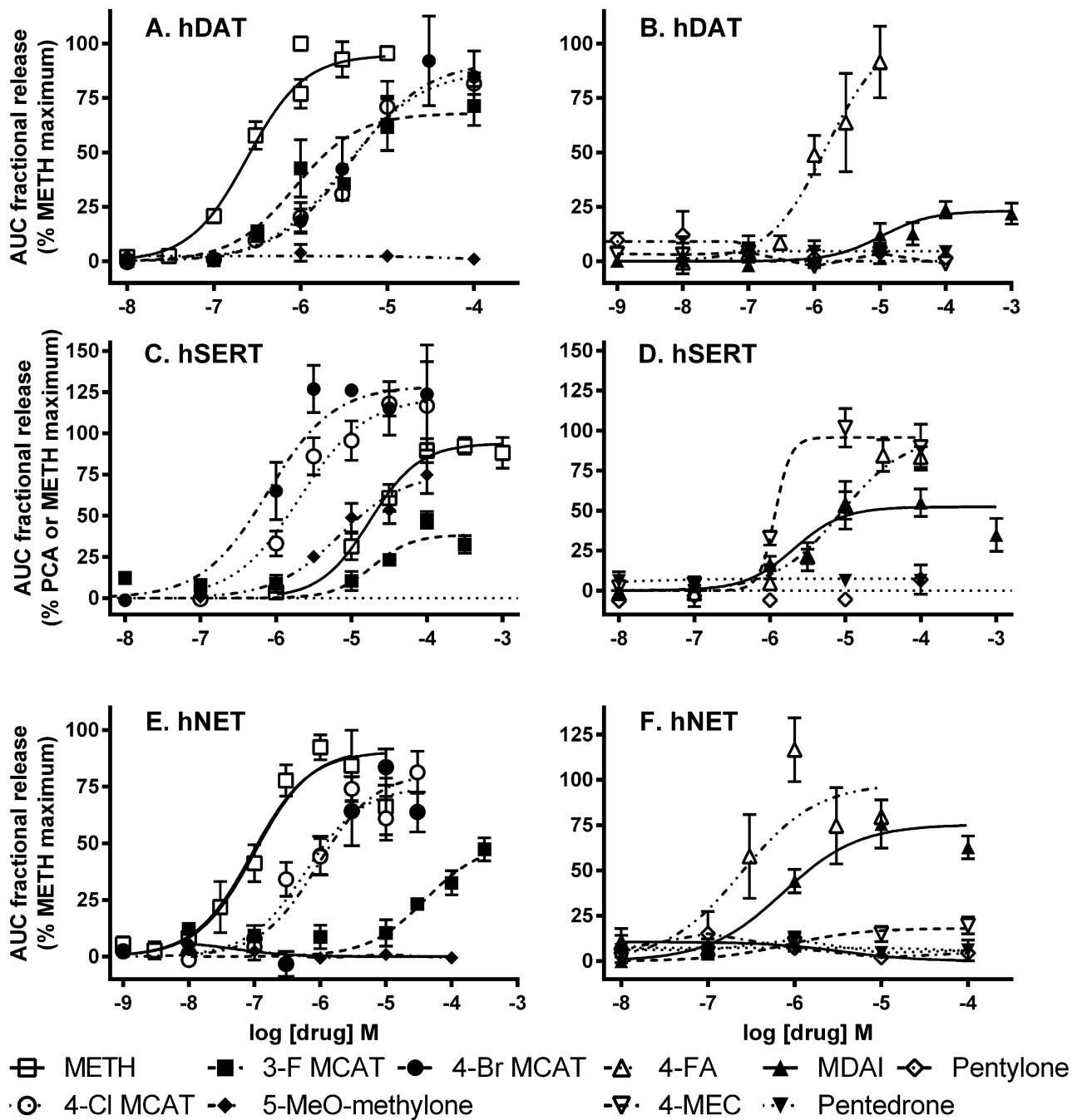


Figure 4

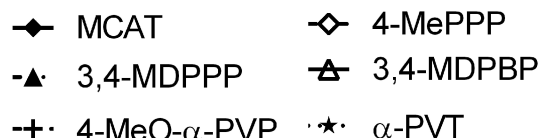
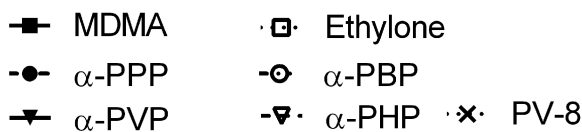
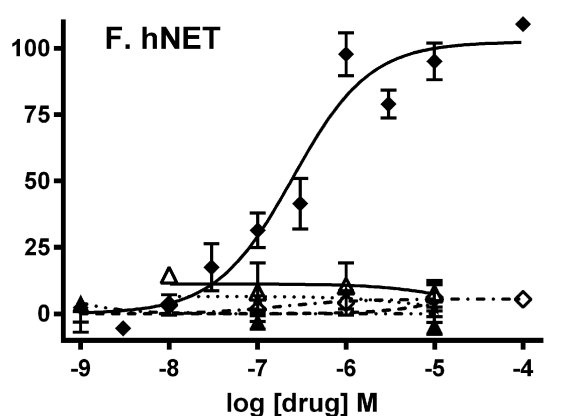
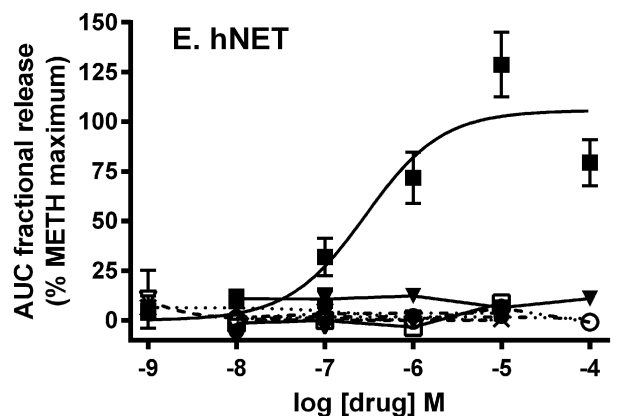
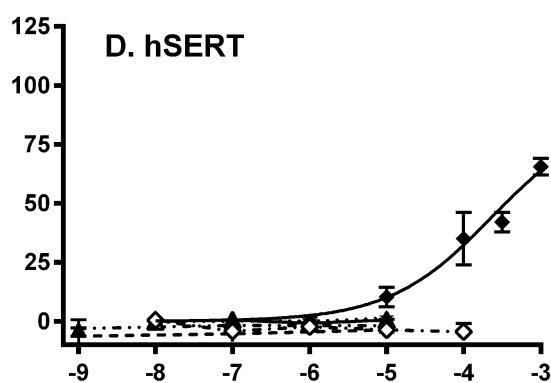
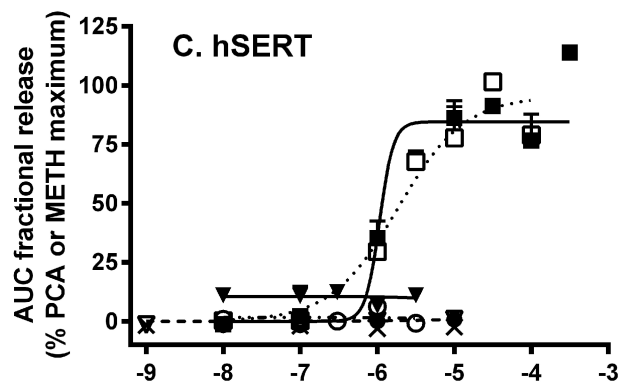
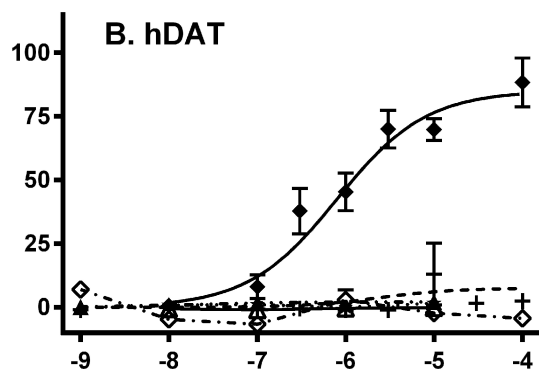
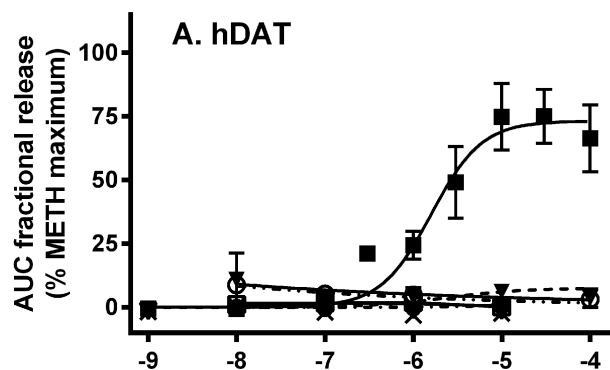


Figure 5

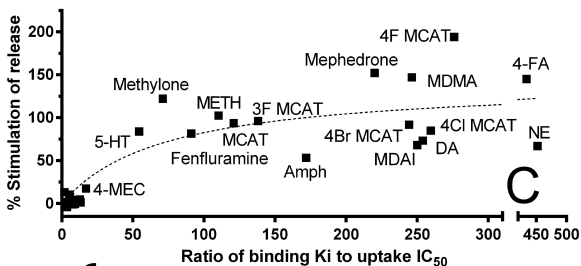
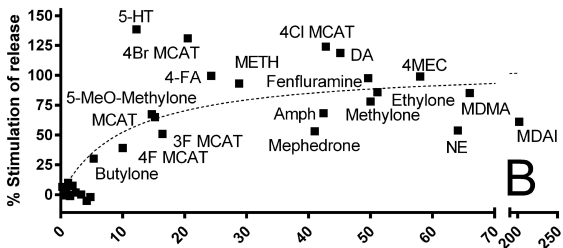
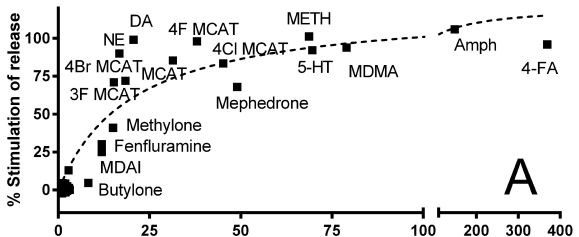


Figure 6

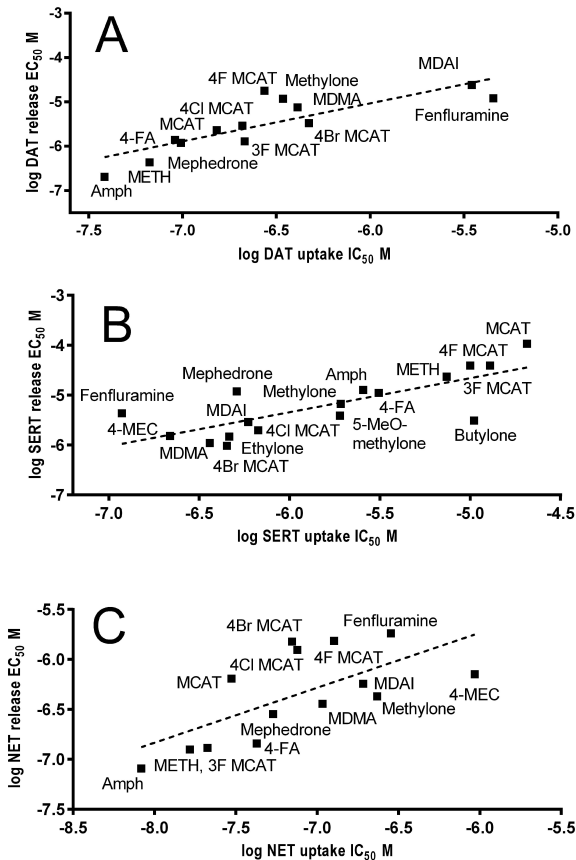


Figure 7