Monoamine transporter and receptor interaction profiles of novel psychoactive substances: para-halogenated amphetamines and pyrovalerone cathinones

Short title: pharmacology of novel psychoactive substances

Anna Rickli,[a] Marius C. Hoener,[b] Matthias E. Liechti[a,*]

[a]Psychopharmacology Research, Division of Clinical Pharmacology and Toxicology, Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland; [b]Pharmaceuticals Division, F. Hoffmann-La Roche Ltd, Basel, Switzerland

* Corresponding author: Prof. Dr. Matthias E. Liechti, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Hebelstrasse 2, Basel, CH-4031, Switzerland. Tel: +41 61 328 68 68; Fax: +41 61 265 45 60; E-mail: matthias.liechti@usb.ch

Word counts: Abstract: 250; Manuscript: 4128

Tables: 2

Figures: 4

References: 48

ABSTRACT

The pharmacology of novel psychoactive substances is mostly unknown. We evaluated the transporter and receptor interaction profiles of a series of para-(4)-substituted amphetamines and pyrovalerone cathinones. We tested the potency of these compounds to inhibit the norepinephrine (NE), dopamine (DA), and serotonin (5-HT) transporters (NET, DAT, and SERT, respectively) using human embryonic kidney 293 cells that express the respective human transporters. We also tested the substance-induced efflux of NE, DA, and 5-HT from monoamine-loaded cells, binding affinities to monoamine receptors, and 5-HT_{2B} receptor activation. Para-(4)-substituted amphetamines, including 4-methylmethcathinone (mephedrone), 4-ethylmethcathinone, 4-fluoroamphetamine, 4-fluoromethamphetamine, 4-fluoromethcatinone (flephedrone), and 4-bromomethcathinone, were relatively more serotonergic (lower DAT:SERT ratio) compared with their analogs amphetamine, methamphetamine, and methcathinone. The 4-methyl, 4-ethyl, and 4-bromo groups resulted in enhanced serotonergic properties compared with the 4-fluoro group. The para-substituted amphetamines released NE and DA. 4-Fluoramphetamine, 4-flouromethamphetamine, 4-methylmethcathinone, and 4-ethylmethcathinone also released 5-HT similarly to 3,4-methylenedioxymethamphetamine. The pyrovalerone cathinones 3,4-methylenedioxypyrovalerone, pyrovalerone, α-pyrrolidinovalerophenone, 3,4-methylenedioxy-α-pyrrolidinopropiophenone, and 3,4-methylenedioxy-α-pyrrolidinobutiophenone potently inhibited the NET and DAT but not the SERT. Naphyrone was the only pyrovalerone that also inhibited the SERT. The pyrovalerone cathinones did not release monoamines. Most of the para-substituted amphetamines exhibited affinity for the 5-HT_{2A} receptor but no relevant activation of the 5-HT_{2B} receptor. All of the cathinones exhibited reduced trace amine receptor 1 binding compared with the non-β-keto-amphetamines. In conclusion, para-substituted amphetamines exhibited enhanced direct and indirect serotonergic agonist properties and are likely associated with more MDMA-like effects. The pharmacological profile of the pyrovalerone cathinones predicts pronounced stimulant effects and high abuse liability.

Keywords: novel psychoactive substances, cathinone, amphetamine, dopamine, serotonin, transporter

1. Introduction

Novel psychoactive substances ("designer drugs") are newly misused psychotropic drugs that may pose a threat to public health that is comparable to previously listed drugs of abuse. Novel psychoactive substances are typically sold through the Internet (i.e., "Internet drugs") and misbranded as "research chemicals," "bath salts,", and "plant food" and labeled "not for human consumption." The substances are typically chemically slightly different from already scheduled drugs to circumvent regulations and are therefore also termed "legal highs." In the last few years, we have seen an unprecedented growth in the number of new psychoactive substances on the illicit drug market. More than 300 novel substances have been detected since 2005 (European Monitoring Center for Drugs and Drug Addiction, 2014a). Currently, more than one new substance is identified in one of the EU countries every week (European Monitoring Center for Drugs and Drug Addiction, 2014a). In most cases, pharmacological data are not available for the newly misused substances. Many novel psychoactive substances are amphetamine derivatives that can be expected to interact with the norepinephrine (NE), dopamine (DA), and serotonin (5-hydroxytryptamine [5-HT]) transporters (NET, DAT, and SERT, respectively) to inhibit monoamine transport or induce transporter-mediated monoamine release. However, chemical substitutions at the amphetamine core structure may significantly alter the absolute or relative potency of these newly designed substances at the NET and DAT relative to the SERT (Baumann et al., 2012; Blough et al., 2014; Cozzi et al., 2013; Eshleman et al., 2013; Iversen et al., 2013; Simmler et al., 2013; Simmler et al., 2014a; Simmler et al., 2014b). Consequently, more noradrenergic and dopaminergic substances may have greater sympathomimetic and reinforcing properties (Simmler et al., 2013). Conversely, more serotonergic substances are likely associated with more MDMA-like properties, including empathogenic effects, serotonin syndrome, and hyperpyrexia (Simmler et al., 2013; Simmler et al., 2014a). Additionally, novel amphetamines may directly activate monoamine receptors. Characterizing the primary pharmacodynamic properties of novel designer amphetamines *in vitro* provides a basis for

further preclinical studies and the evaluation of potential clinical effects, abuse potential, and acute toxicity of these novel substances. Such data are useful for clinical toxicologists and regulatory agencies for scheduling purposes. Therefore, the aim of the present study was to determine the effects of a series of para-(4)-substituted amphetamines and of a series of pyrovalerone cathinones on monoamine uptake and release and interactions with various monoamine receptors.

Para-(4)-phenyl-substituted amphetamines, which have emerged in recent years, include 4-methylmethcathinone (mephedrone) and 4-ethylmethcathinone and particularly several para-halogenated compounds, including 4-fluoroamphetamine, 4-fluoromethamphetamine, 4-fluoromethcatinone (flephedrone), and 4-bromomethcathinone. 4-Methylmethcathinone has been the most popular and still is a very commonly misused cathinone in the EU (Elliott and Evans, 2014; Helander et al., 2014; Rust et al., 2012; Winstock et al., 2011). 4-Ethylmethcatinone was detected in 2011 in the EU (European Monitoring Center for Drugs and Drug Addiction, 2011), and its use is discussed in Internet user forums. Similarly, the use of 4-bromomethcathinone is also discussed in user forums, but no scientific data are available. 4-Fluoroamphetamine appeared in 2007 in the EU, followed later by 4-fluoromethamphetamine and 4-fluoroephedrine. 4-Fluoroephedrine may serve as a precursor for the synthesis of 4-fluoromethamphetamine. 4-Fluoroamphetamine and 4-fluoromethamphetamine have also been detected in patients with acute toxicity associated with novel psychoactive substances and forensic cases (Helander et al., 2014; Johansen and Hansen, 2012; Rohrich et al., 2012; Rust et al., 2012). Users report that the subjective effects of 4-methlylmethcathinone (Carhart-Harris et al., 2011) and 4-fluoroamphetamine (Erowid, 2014) are comparable to those of MDMA. Pharmacological information is available only for some of these novel substances, including 4-methylmethcatinone (Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013), 4-fluoroamphetamine (Marona-Lewicka et al., 1995), and 4-fluoromethcathinone (Eshleman et al., 2013; Simmler et al., 2013). Because 4-fluoroamphetamine and MDMA are relatively more serotonergic than amphetamine and methamphetamine (Marona-Lewicka et al., 1995; Simmler et al., 2013), we hypothesized that

a substitution at the 4-position as a characteristic of these novel para-substituted substances would also result in a shift toward more serotonergic than dopaminergic pharmacology. Thus, such para-substituted substances may also be designed to mimic the effects of MDMA.

Pyrovalerone cathinones include 3,4,-methylenedioxypyrovalerone (MDPV), pyrovalerone, α-pyrrolidinovalerophenone (α-PVP), naphyrone, 3,4-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP), 3,4-methylenedioxy-α-pyrrolidinobutiophenone (MDPBP), α-pyrrolidinopropiophenone (α-PPP), and α-pyrrolidinobutiophenone (α-PBP). All of these cathinones are characterized by a pyrrolidine ring structure, making them different structurally and possibly also pharmacologically from other synthetic cathinones (Marusich et al., 2014; Simmler et al., 2013). Among the pyrovalerone cathinones, MDPV is currently the most widely detected and used, both in the EU (European Monitoring Center for Drugs and Drug Addiction, 2014b; Helander et al., 2014; Zuba and Byrska, 2013) and US (Leffler et al., 2014; Marinetti and Antonides, 2013; Spiller et al., 2011). In fact, MDPV has become the most frequently detected and used of all cathinones ("bath salts") in some EU countries (Helander et al., 2014; Zuba and Byrska, 2013) and the US (Leffler et al., 2014). More recently, a second generation of MDPV-like cathinones, including α-PVP, MDPPP, and MDPBP, has been detected and/or used in several EU countries (Eiden et al., 2013; Helander et al., 2014; Westphal et al., 2011; Zuba and Byrska, 2013) and the US (Elliott and Evans, 2014; Smollin et al., 2011; Thornton et al., 2012). MDPV has been associated with severe clinical toxicity (Spiller et al., 2011) and a high potential for addiction (Aarde et al., 2013). Similarly, α-PVP has recently been associated with cases of severe acute psychosis and cardiac arrest (Eiden et al., 2013). Pharmacologically, both MDPV and α-PVP are very potent inhibitors of the NET and DAT but not SERT (Baumann et al., 2013; Marusich et al., 2014; Meltzer et al., 2006; Simmler et al., 2013). Of the second generation MDPV analogs, α-PPP and α-PBP also inhibit the NET and DAT similarly to MDPV (Marusich et al., 2014), but no data are available on MDPBP and MDPPP. We hypothesized that these and other cathinones with a pyrovalerone structure would inhibit the NET and DAT but not SERT, similar to MDPV (Marusich et al., 2014; Meltzer et al., 2006; Simmler et al., 2013). Naphyrone also

potently inhibits the SERT, unlike other pyrovalerone cathinones, and this exemplifies the necessity to pharmacologically assess each substance individually to avoid drawing false conclusions from structural relationships with previously assessed analogs. We predicted that these pyrovalerone cathinones are distinct from other cathinones, in which they are pure uptake inhibitors and do not act as substrate releasers as previously shown for MDPV (Baumann et al., 2013; Simmler et al., 2013).

We tested whether the substances inhibit the human NET, DAT, and SERT. We also determined the transporter-mediated release of NE, DA, and 5-HT and characterized the binding affinities of the compounds for monoamine transporters, α_1- and α_2-adrenergic receptors, dopamine D_1-D_3 receptors, serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, the histamine H_1 receptor, and trace amine-associated receptor 1 ($TAAR_1$). For example, 5-HT_{2A} receptors mediate the effects of hallucinogens (Nichols, 2004) and $TAAR_1$ play a role in the addictive properties of psychoactive substances (Pei et al., 2014). Furthermore, some novel psychoactive substances have been reported to bind to the serotonin 5-HT_{2B} receptor (Iversen et al., 2013), which has been implicated in endocardial fibrosis induced by serotonergic substances. Therefore, we also tested functional activity at the 5-HT_{2B} receptor.

Some of the substances, including MDMA, amphetamine, methamphetamine, methcathinone, mephedrone, flephedrone, MDPV, naphyrone, and pyrovalerone, have previously been characterized using the same assays as those used in the present study (Simmler et al., 2013), but we retested them herein because of their structural similarity to the other substances that were evaluated, to our knowledge, for the first time.

2. Methods

2.1. Drugs

MDMA, amphetamine, methamphetamine, methcathinone, 4-methylmethcathinone, 4-fluoromethcathione, 4-fluoroamphetamine, 4-fluoroephedrine, ephedrine, MDPBP, MDPPP, MDPV, pyrovalerone, and α-PVP were purchased from Lipomed (Arlesheim, Switzerland). 4-Fluoromethamphetamine, 4-etylmethcathinone, and 4-bromomethcathinone

were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Naphyrone was synthesized as previously described (Simmler et al., 2013). All of the drugs were obtained as racemic hydrochloride salts, with the exception of ephedrine, amphetamine, and methamphetamine, for which the (-)-enantiomer was used. Purity was at least 98% for all of the substances. Radiochemicals (tritium isotopes) were obtained from Anawa (Wangen, Switzerland) or Perkin Elmer (Schwerzenbach, Switzerland), with the exception of [^{3}H]RO5166017, which was synthesized at Roche (Basel, Switzerland).

2.2. Monoamine uptake transport inhibition

Inhibition of the NET, SERT, and DAT was assessed in human embryonic kidney 293 (HEK 293) cells that stably expressed the human NET, SERT, and DAT (Tatsumi et al., 1997) as previously described (Hysek et al., 2012). Cultured cells were detached and resuspended in uptake buffer. We incubated the cells with various concentrations of the test compounds and the vehicle control for 10 min and then added [^{3}H]DA, [^{3}H]NE, and [^{3}H]5-HT (5 nM final concentration) to initiate uptake transport of the labeled monoamines at room temperature. Uptake was stopped after 10 min by separation of the cells from the buffer by rapid high-speed centrifugation through silicone oil (Hysek et al., 2012). The uptake times were based on previous kinetic evaluations that showed that uptake is complete after 5 min (Hysek et al., 2012). The centrifugation tubes were frozen in liquid nitrogen and cut to separate the cell pellet from the silicone oil and assay buffer layers. The cell pellet was then lysed. Scintillation fluid was added, and radioactivity was counted on a beta-counter. Nonspecific uptake was determined for each experiment in the presence of 10 μM fluoxetine for SERT cells, 10 μM nisoxetine for NET cells, and 10 μM mazindol for DAT cells and subtracted from the total counts to yield specific uptake (100%). Nonspecific uptake was < 15% of total uptake. The data were fit by non-linear regression to variable-slope sigmoidal dose-response curves, and IC_{50} values were calculated using Prism (GraphPad, San Diego, CA, USA). DAT:SERT inhibition ratios were calculated as 1/DAT IC_{50}:1/SERT IC_{50}. The

DAT:SERT inhibition ratio is useful for predicting the characteristics of the psychoactive effects of novel psychoactive substances (Baumann et al., 2011; Simmler et al., 2013; Wee et al., 2005). Higher relative potency at the DAT may indicate a higher abuse potential, whereas relatively increased activity of the 5-HT system is linked to a reduction of abuse potential and more MDMA-like psychotropic effects (Wee et al., 2005). Stimulant amphetamines, such as methamphetamine, have a DAT:SERT inhibition ratio > 10, whereas MDMA and other substances with MDMA-like psychotropic effects have a DAT:SERT inhibition ratio close to 0.1 (Baumann et al., 2012; Simmler et al., 2013; Simmler et al., 2014a; Simmler et al., 2014b).

2.3. Transporter-mediated monoamine release

We studied the effects of 100 µM of the test compounds on transporter-mediated NE, 5-HT, and DA efflux in HEK 293 cells that overexpressed the respective human monoamine transporter as previously reported in detail (Simmler et al., 2013). Briefly, we preloaded the cells by incubating SERT cells with 10 nM [^{3}H]5-HT, DAT cells with 10 nM [^{3}H]DA and 1 µM unlabeled DA, and NET cells with 10 nM [^{3}H]NE and 10 µM unlabeled NE for 20 min. The cells were then washed twice, and release was induced by adding 1000 µl of release buffer that contained the test compounds at concentrations of 100 µM. We incubated the SERT and DAT cells for 15 min and NET cells for 45 min at 37°C with shaking at 300 rotations per minute on a rotary shaker. The release times were based on kinetic evaluation of the release-over-time curves for MDMA. After 15 min for [^{3}H]5-HT and [^{3}H]DA and 45 min for [^{3}H]NE, a sufficient amount of radioactivity was released to allow for comparisons with the control conditions. We then stopped release by removing the buffer and gently washing the cells twice with cold buffer. We quantified the radioactivity that remained in the cells. Nonspecific "pseudo-efflux," which arises from nonspecific substrate release and subsequent reuptake inhibition (Scholze et al., 2000), was assessed for each experiment using the transporter inhibitors nisoxetine (NET cells), citalopram (SERT cells), and mazindol (DAT cells) at 10 µM as negative control conditions. We then used analysis of variance followed by

the Least Significant Difference test to compare substance-induced monoamine release with nisoxetine, citalopram, and mazindol as negative controls. Substances that induced significantly higher monoamine efflux at 100 μM compared with the respective transporter inhibitors, which induced slight nonspecific release, were considered monoamine releasers.

2.4. Radioligand binding assays

The radioligand binding assays were performed as described previously (Hysek et al., 2012; Revel et al., 2011; Simmler et al., 2013). Briefly, membrane preparations of HEK 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective transporters (Tatsumi et al., 1997) or receptors (human genes plus $TAAR_1$ rat and mouse genes; Revel et al., 2011) were incubated with the radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between the total binding and nonspecific binding determined in the presence of selected competitors in excess. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]-nisoxetine and indatraline (NET), [^{3}H]citalopram and indatraline (SERT), [^{3}H]WIN35,428 and indatraline (DAT), [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin and indatraline (5-HT_{1A} receptor), [^{3}H]ketanserin and spiperone (5-HT_{2A} receptor), [^{3}H]mesulergine and mianserin (5-HT_{2C} receptor), [^{3}H]prazosin and risperidone (α_1 adrenergic receptor), [^{3}H]rauwolscine and phentolamine (α_2 adrenergic receptor), [^{3}H]SCH 23390 and butaclamol (D_1 receptor), [^{3}H]spiperone and spiperone (D_2 and D_3 receptors), [^{3}H]pyrilamine and clozapine (histaminergic H_1 receptor), and [^{3}H]RO5166017 and RO5166017 ($TAAR_1$). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using three to five independent 10-point concentration-response curves for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation. As indicated in Table 2, previously published binding affinity data for some of the substances are included for comparative purposes (Simmler et al., 2013).

2.5. Functional serotonin 5-HT_{2B} receptor activity

The 5-HT_{2B} receptor functional assay was performed as previously described (Jensen et al., 2008). Briefly, human 5-HT_{2B} receptor-expressing HEK 293 cells were incubated at 37ºC in 96-well plates coated with poly-D-lysine. The growth medium was removed by snap inversion, and 100 µl of Fluo-4 solution (calcium indicator; Molecular Probes) was added. The plates were incubated for 45 min at 31ºC. The Fluo-4 solution was removed by snap inversion, and 100 µl of Fluo-4 solution was added for the second time. The cells were then incubated for another 45 min at 31ºC. Immediately before testing, the cells were washed with HBSS (Gibco) and 20 mM HEPES (assay buffer; Gibco) using an EMBLA cell washer, and 100 µl assay buffer was added. The plate was placed in a fluorescence imaging plate reader (FLIPR), and 25 µl of the test substances diluted in assay buffer was added on line. The increase in fluorescence was then measured. EC_{50} values were derived from the concentration-response curves using nonlinear regression. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

2.6. Cytotoxicity

Cell membrane integrity during uptake and release testing was verified after 4 h treatment with each of the drugs (100 µM) using the ToxiLight BioAssay (Lonza, Basel, Switzerland).

3. Results

3.1. Monoamine uptake transporter inhibition

The monoamine transporter inhibition profiles are shown Fig. 2, and the corresponding IC_{50} values and DAT:SERT inhibition ratios are listed in Table 1. In all cases, the para-(4) substitution (Fig. 1A) reduced the potency of the amphetamines to inhibit both the NET and DAT compared with the non-para-(4)-substituted amphetamines (Table 1). In contrast, the potency to inhibit the SERT increased for all of the substituted amphetamines,

with the exception of 4-fluoromethcathinone compared with methcathinone (Table 1). As a result, the para-substituted substances were all relatively more serotonergic than dopaminergic compared with their parent compounds, reflected by their lower DAT:SERT inhibition ratios (Table 1). This was also evident for 4-fluoromethcathinone and methcathinone, despite equal SERT inhibition potencies. In the case of 4-fluoroephedrine, 4-methylmethcathinone, 4-ethylmethcathinone, and 4-bromomethcathinones, the para substitution left-shifted the SERT inhibition curves over the DAT inhibition curves (DAT:SERT inhibition ratios < 1), resulting in monoamine transporter inhibition profiles that were more similar to MDMA and less similar to the parent compounds (methcathinone and ephedrine; Fig. 2). In contrast, all of the pyrovalerone cathinones (Fig. 1B) were very potent catecholamine transporter (NET and DAT) inhibitors with very low serotonergic activity, reflected by very high DAT:SERT inhibition ratios (Table 1). One exception was naphyrone, which also inhibited the SERT at submicromolar concentrations. The 3,4-methylene ring substitution that is found in MDMA and MDPV increased serotonergic activity compared with the non-substituted compounds methamphetamine and α-PVP, respectively. Similarly, para-methylation in pyrovalerone increased the serotonergic property of the compound compared with α-PVP. However, in the case of the pyrovalerones (MDPV and pyrovalerone), SERT inhibition potency was very low, even in the presence of these substitutions. In fact, all of the pyrovalerone cathinones (Fig. 1B) did not appear to interact with the SERT at submicromolar concentrations, with the exception of naphyrone.

3.2. Transporter-mediated monoamine release

Monoamine release is shown in Fig. 4. All of the para-substituted amphetamine derivatives released NE and DA similarly to their non-substituted classic analogs amphetamine, methamphetamine, and methcathinone. Additionally, 4-fluoramphetamine, 4-flouromethamphetamine, 4-methylmethcathinone, 4-ethylmethcathinone, amphetamine, and methamphetamine significantly released 5-HT similarly to the classic 5-HT releaser MDMA. 4-Fluoromethcathinone, 4-bromomethcathinone, methcathinone, and ephedrine only released

catecholamines and not 5-HT, whereas 5-fluoroephedrine released only NE. The pyrovalerone cathinones did not release monoamines (Fig. 4) and thus acted as pure and potent uptake inhibitors (Table 1).

3.3. Binding affinities

The monoamine transporter and receptor binding affinities are shown in Tables 2 and 3. The pyrovalerone cathinones exhibited high affinity for the DAT and mostly also for the NET, consistent with their high DAT and NET blocking potency (Table 1). Most of the para-substituted amphetamines exhibited affinity for the serotonin 5-HT_{2A} receptor in the low micromolar range, similar to MDMA and dissimilar to amphetamine and methamphetamine (Table 3). The cathinones (β-keto-amphetamines) showed lower binding affinity for $TAAR_1$ compared with the non-β-keto-amphetamines (Table 2).

3.4. Functional activity at serotonin 5-HT_{2B} receptors

None of the substances tested exhibited relevant activation of the 5-HT_{2B} receptor (Table 3). Amphetamine was the most potent activator with an IC_{50} of only 9.7 μM. However, there was only very low efficacy of 9%.

3.5. Cytotoxicity

None of the drugs showed cytotoxicity at the highest concentration tested in the functional assays.

4. Discussion

The goal of the present study was to describe the mechanism of action of two series of novel psychoactive substances: para-(4)-substituted (mostly halogenated) amphetamines and pyrovalerone cathinones. All of the para-(4)-substituted amphetamines evaluated in this study exhibited more serotonergic properties than their non-substituted amphetamine analogs. In particular, 4-bromomethcatinone, 4-ethylmethcathinone, and 4-methylmethcathinone were

more potent SERT inhibitors than DAT inhibitors, similar to MDMA. These findings are consistent with previous studies that reported an increase in serotonergic potency in para-ring-substituted amphetamines or phenethylamines (Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013). Para-methylation (as in 4-methylmethcathinone) reduced the potency of DAT and increased the potency of SERT inhibition compared with methcathinone, consistent with previous studies (Eshleman et al., 2013; Simmler et al., 2013). Similarly, the para-methylation of amphetamine has previously been shown to result in reduced DAT inhibition and increased SERT inhibition (Wee et al., 2005). The para-flourination of ephedrine, amphetamine, and methamphetamine resulted in relatively more serotonergic properties, reflected by lower DAT:SERT inhibition ratios compared with the non-substituted analogs in the present study, confirming data on 4-fluoroamphetamine in rat brain synaptosomes (Marona-Lewicka et al., 1995; Wee et al., 2005) and 4-flouromethcathinone in human cell assays (Eshleman et al., 2013; Simmler et al., 2013). The presence of an ethyl or methyl group in the para position resulted in more pronounced serotonergic properties compared with a fluoro group, consistent with previous data on 4-methcathinone and fluoromethcathinone *vs.* cathinone (Simmler et al., 2013) and 4-methylamphetamine and 4-fluoroamphetamine *vs.* amphetamine (Wee et al., 2005). With regard to haloamphetamines, para substitution with fluoride only moderately increased the relative serotonergic properties (DAT:SERT inhibition ratio) of several compounds in the present study (5- to 15-fold), whereas bromide was more effective (48-fold) and close to chloride (64-fold; Marona-Lewicka et al., 1995) but still less effective than iodine (548-fold; Marona-Lewicka et al., 1995). Finally, other para-substituted amphetamines, including 4-methylthioamphetamine, para-methoxyamphetamine, para-methoxymethamphetamine, methedrone, and 4-trifluoromethylmethcathinone, have previously been shown to preferentially interact with the SERT and NET over the DAT (Cozzi et al., 2013; Simmler et al., 2014a). The entactogenic effects of the popular recreational drug MDMA depend on its serotonergic effects (Hysek et al., 2012). Consequently, substances that predominantly increase 5-HT can be expected to produce MDMA-like subjective effects. Additionally, the serotonergic properties of these

substances likely increase the risk for serotonergic toxicity, including serotonin syndrome and hyperthermia (Liechti et al., 2005; Simmler et al., 2011). In behavioral drug discrimination studies, 4-fluoroamphetamine, which is only moderately more serotonergic than amphetamine, is similar to amphetamine (Marona-Lewicka et al., 1995). In contrast, 4-chloroamphetamine and 4-iodoamphetamine, which are more serotonergic (Marona-Lewicka et al., 1995), were behaviorally similar to MDMA-like drugs (Marona-Lewicka et al., 1995). 4-Methylmethcathinone exhibited a DAT:SERT inhibition ratio more similar to MDMA than to amphetamine in the present study but was more dopaminergic in other *in vitro* studies (Eshleman et al., 2013; Iversen et al., 2013; Simmler et al., 2013). *In vivo*, mephedrone has been shown to increase DA similarly to amphetamine (Kehr et al., 2011) and 5-HT similarly to MDMA (Baumann et al., 2012; Kehr et al., 2011). Behaviorally, mephedrone was similar to MDMA (Baumann et al., 2012). The subjective effects of 4-methylmethcathinone are also reported to be similar to MDMA (Carhart-Harris et al., 2011) but also to cocaine (Winstock et al., 2011). Thus, mephedrone appears to exhibit both empathogenic and stimulant properties.

In the present study, we also characterized the widely used cathinone MDPV, its analogs pyrovalerone and α-PVP, and two novel and similar compounds, MDPBP and MDPPP. These pyrovalerone cathinones all potently inhibited both the NET and DAT, confirming previous studies with MDPV (Baumann et al., 2013; Eshleman et al., 2013; Meltzer et al., 2006; Simmler et al., 2013), pyrovalerone (Meltzer et al., 2006; Simmler et al., 2013), and α-PVP (Marusich et al., 2014; Meltzer et al., 2006). Very recently, α-PBP and α-PPP have similarly been shown to be selective and potent catecholamine uptake inhibitors (Marusich et al., 2014). Additionally, none of the pyrovalerone derivatives tested in the present study released monoamines, as expected with regard to earlier findings with pyrovalerones (Baumann et al., 2013; Simmler et al., 2013). The pyrovalerone cathinones, which contain a pyrrolidine ring, likely represent a subgroup of cathinones that are mechanistically distinct from most other cathinones that also release monoamines similarly to the classic amphetamines (Baumann et al., 2012; Eshleman et al., 2013; Simmler et al., 2013). The pyrovalerones with the longest α-side chain, including α-PVP, MDPV, and pyrovalerone, were the most potent DAT and NET

inhibitors, followed by α-PBP and MDPBP and by α-PPP and MDPPP, respectively (Marusich et al., 2014, and the present study). As shown for the para-substituted amphetamines in the first series of this study, the para-(4) substitution in pyrovalerone or the 3,4-methylenedioxy substitution in MDPV, MDPBP, and MDPPP increased the absolute and relative serotonergic potency of the substances compared with the non-substituted parent drug α-PVP in the present study or compared with α-PBP and α-PPP (Marusich et al., 2014). However, serotonergic activity remained low for all these substances. Interestingly, naphyrone was the only pyrovalerone cathinone that also potently inhibited the SERT, confirming previous studies (Eshleman et al., 2013; Iversen et al., 2013; Meltzer et al., 2006; Simmler et al., 2013). With the exception of naphyrone, a hallmark of all other pyrovalerone cathinones is that they very potently inhibit the DAT but not SERT. Dopamine transporter-selective over SERT-selective amphetamines produce more stimulant and abuse-related effects than substances with a mixed action at the DAT and SERT (Baumann et al., 2011; Wee et al., 2005). Accordingly, the very high DAT:SERT inhibition ratio induced by the pyrovalerone cathinones predicts particularly pronounced stimulant and addictive properties for this class of substances. In fact, MDPV and α-PVP are considered highly addictive (Aarde et al., 2013; Baumann et al., 2013; Watterson et al., 2014). Additionally, intoxication with MDPV, naphyrone, and α-PVP is associated with pronounced agitation, prolonged insomnia, psychotic symptoms, tachycardia, and cardiac arrest (Derungs et al., 2011; Eiden et al., 2013; European Monitoring Center for Drugs and Drug Addiction, 2014b; Spiller et al., 2011). Similar sympathetic stimulation with wild agitation and hallucinations has also been described with MDPPP (Smollin et al., 2011). One feature of intoxication with pyrovalerone cathinones is their long duration of insomnia, which can last up to several days (Derungs et al., 2011; Eiden et al., 2013). The long duration of action could be linked to the high potency of the drugs and an increased risk of overdosing. Additionally, the pyrovalerones are all highly lipophilic substances with associated high brain penetration (Simmler et al., 2013) and a high volume of distribution, resulting in longer plasma and tissue half-lives (Derungs et al., 2011).

Most para-substituted amphetamines in this series exhibited direct affinity for the serotonin 5-HT_{2A} receptor. The 5-HT_{2A} receptor mediates the hallucinogenic effects of hallucinogens (Nichols, 2004) and also the hallucinogen-like perceptual changes associated with higher doses of MDMA (Liechti et al., 2000). Accordingly, these substances act as indirect and direct serotonergic agonists and may induce perceptual alterations. None of the compounds showed relevant activity as agonists at the 5-HT_{2B} receptor. In contrast, other structurally related novel psychoactive substances (benzofurans) have been shown to activate the 5-HT_{2B} receptor (Iversen et al., 2013), which has been suggested to be associated with an increased risk of endocardial fibrosis (Iversen et al., 2013). Thus, our data do not indicate a risk for endocardial fibrosis for the substances tested in this series. We found that amphetamines consistently showed higher $TAAR_1$ binding affinities compared with the cathinones and ephedrins that carry a β-keto or β-hydroxy group, respectively. Consistently, other cathinones did not exhibit relevant $TAAR_1$ binding (Simmler et al., 2013; Simmler et al., 2014a). We also found that amphetamines not only bind to rodent receptors but also human $TAAR_1$. In rodents, non-β-keto amphetamines inhibit their own stimulant effects via $TAAR_1$ activation (Di Cara et al., 2011). The lack of this $TAAR_1$-mediated "auto-inhibition" with the cathinones may contribute to more stimulant-like and addictive properties of this new class of novel psychoactive substances compared with traditional amphetamines (Simmler et al., 2013).

A particular strength of the present study was the inclusion a relatively large number of substances and comprehensive characterization at many targets. Other studies typically only assessed monoamine uptake inhibition and not substrate release or binding affinities for other monoamine receptors. Additionally, in the transporter inhibition assays, we also included high concentrations when needed to allow for better characterization of full dose-response curves and determination of higher IC_{50} values.

The present study also has limitations. For example, we did not investigate the effects of the drugs on intracellular targets, such as the vesicular monoamine transporter or monoamine oxidase, which are affected by amphetamines (Eshleman et al., 2013). We also focused on

pharmacodynamics *in vitro*. Many additional factors, such as brain penetration, metabolism, and pharmacokinetics, also play a role in the clinical effects of these substances, which require further study *in vivo*.

5. Conclusion

Para-(4)-substituted amphetamines are more serotonergic than their non-substituted analogs, likely resulting in more MDMA-like serotonergic subjective and acute toxic effects. Pyrovalerone cathinones are potent NET and DAT inhibitors that are likely associated with significant stimulant-type effects and toxicity and a high risk of addiction.

Conflict of interest

The authors do not have any conflicts of interest to declare for this work.

Acknowledgements

This work was supported by the Federal Office of Public Health (no. 13.006497) and the Translational Medicine Hub Innovation Fund of F. Hoffmann-La Roche and the University of Basel. The authors thank Daniele Buchy for performing the experiments, Linda Simmler for critical comments on the manuscript, and Michael Arends for text editing.

References

Aarde, S.M., Huang, P.K., Creehan, K.M., Dickerson, T.J., Taffe, M.A., 2013. The novel recreational drug 3,4-methylenedioxypyrovalerone (MDPV) is a potent psychomotor stimulant: self-administration and locomotor activity in rats. Neuropharmacology 71, 130-140.

Baumann, M.H., Ayestas, M.A., Jr., Partilla, J.S., Sink, J.R., Shulgin, A.T., Daley, P.F., Brandt, S.D., Rothman, R.B., Ruoho, A.E., Cozzi, N.V., 2012. The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. Neuropsychopharmacology 37, 1192-1203.

Baumann, M.H., Clark, R.D., Woolverton, W.L., Wee, S., Blough, B.E., Rothman, R.B., 2011. In vivo effects of amphetamine analogs reveal evidence for serotonergic inhibition of mesolimbic dopamine transmission in the rat. J Pharmacol Exp Ther 337, 218-225.

Baumann, M.H., Partilla, J.S., Lehner, K.R., Thorndike, E.B., Hoffman, A.F., Holy, M., Rothman, R.B., Goldberg, S.R., Lupica, C.R., Sitte, H.H., Brandt, S.D., Tella, S.R., Cozzi, N.V., Schindler, C.W., 2013. Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacology 38, 552-562.

Blough, B.E., Landavazo, A., Decker, A.M., Partilla, J.S., Baumann, M.H., Rothman, R.B., 2014. Interaction of psychoactive tryptamines with biogenic amine transporters and serotonin receptor subtypes. Psychopharmacology, in press.

Carhart-Harris, R.L., King, L.A., Nutt, D.J., 2011. A web-based survey on mephedrone. Drug Alcohol Depend 118, 19-22.

Cozzi, N.V., Brandt, S.D., Daley, P.F., Partilla, J.S., Rothman, R.B., Tulzer, A., Sitte, H.H., Baumann, M.H., 2013. Pharmacological examination of trifluoromethyl ring-substituted methcathinone analogs. Eur J Pharmacol 699, 180-187.

Derungs, A., Schietzel, S., Meyer, M.R., Maurer, H.H., Krahenbuhl, S., Liechti, M.E., 2011. Sympathomimetic toxicity in a case of analytically confirmed recreational use of naphyrone (naphthylpyrovalerone). Clin Toxicol (Phila) 49, 691-693.

Di Cara, B., Maggio, R., Aloisi, G., Rivet, J.M., Lundius, E.G., Yoshitake, T., Svenningsson, P., Brocco, M., Gobert, A., De Groote, L., Cistarelli, L., Veiga, S., De Montrion, C., Rodriguez, M., Galizzi, J.P., Lockhart, B.P., Coge, F., Boutin, J.A., Vayer, P., Verdouw, P.M., Groenink, L., Millan, M.J., 2011. Genetic deletion of trace amine 1 receptors reveals their role in auto-inhibiting the actions of ecstasy (MDMA). J Neurosci 31, 16928-16940.

Eiden, C., Mathieu, O., Cathala, P., Debruyne, D., Baccino, E., Petit, P., Peyriere, H., 2013. Toxicity and death following recreational use of 2-pyrrolidino valerophenone. Clin Toxicol (Phila) 51, 899-903.

Elliott, S., Evans, J., 2014. A 3-year review of new psychoactive substances in casework. Forensic Sci Int 243C, 55-60.

European Monitoring Center for Drugs and Drug Addiction, 2011. EMCDDA–Europol 2011 Annual Report on the implementation of Council Decision 2005/387/JHA, Lisbon, Portugal.

European Monitoring Center for Drugs and Drug Addiction, 2014a. European Drug Report 2014. European Monitoring Center for Drugs and Drug Addiction. www.emcdda.europa.eu

European Monitoring Center for Drugs and Drug Addiction, 2014b. Report on the risk assessment of 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one (MDPV) in the framework of the Council Decision on new psychoactive substances. EMCDDA, Lisbon, Portugal.

Erowid, 2014. 4-fluoroamphetamine reports. www.erowid.org/chemicals/4_fluoroamphetamine

Eshleman, A.J., Wolfrum, K.M., Hatfield, M.G., Johnson, R.A., Murphy, K.V., Janowsky, A., 2013. Substituted methcathinones differ in transporter and receptor interactions. Biochem Pharmacol 85, 1803-1815.

Helander, A., Backberg, M., Hulten, P., Al-Saffar, Y., Beck, O., 2014. Detection of new psychoactive substance use among emergency room patients: results from the Swedish STRIDA project. Forensic Sci Int 243C, 23-29.

Hysek, C.M., Simmler, L.D., Nicola, V., Vischer, N., Donzelli, M., Krähenbühl, S., Grouzmann, E., Hoener, M.C., Liechti, M.E., 2012. Duloxetine inhibits effects of MDMA ("ecstasy") in vitro and in humans in a randomized placebo-controlled laboratory study. PLoS One 7, e36476.

Iversen, L., Gibbons, S., Treble, R., Setola, V., Huang, X.P., Roth, B.L., 2013. Neurochemical profiles of some novel psychoactive substances. Eur J Pharmacol 700, 147-151.

Jensen, N.H., Rodriguiz, R.M., Caron, M.G., Wetsel, W.C., Rothman, R.B., Roth, B.L., 2008. *N*-desalkylquetiapine, a potent norepinephrine reuptake inhibitor and partial 5-HT_{1A} agonist, as a putative mediator of quetiapine's antidepressant activity. Neuropsychopharmacology 33, 2303-2312.

Johansen, S.S., Hansen, T.M., 2012. Isomers of fluoroamphetamines detected in forensic cases in Denmark. Int J Legal Med 126, 541-547.

Kehr, J., Ichinose, F., Yoshitake, S., Goiny, M., Sievertsson, T., Nyberg, F., Yoshitake, T., 2011. Mephedrone, compared to MDMA (ecstasy) and amphetamine, rapidly increases both dopamine and serotonin levels in nucleus accumbens of awake rats. Br J Pharmacol 164, 1949-1958.

Leffler, A.M., Smith, P.B., de Armas, A., Dorman, F.L., 2014. The analytical investigation of synthetic street drugs containing cathinone analogs. Forensic Sci Int 234, 50-56.

Liechti, M.E., Kunz, I., Kupferschmidt, H., 2005. Acute medical problems due to Ecstasy use: case-series of emergency department visits. Swiss Med Wkly 135, 652-657.

Liechti, M.E., Saur, M.R., Gamma, A., Hell, D., Vollenweider, F.X., 2000. Psychological and physiological effects of MDMA ("Ecstasy") after pretreatment with the 5-HT_2 antagonist ketanserin in healthy humans. Neuropsychopharmacology 23, 396-404.

Marinetti, L.J., Antonides, H.M., 2013. Analysis of synthetic cathinones commonly found in bath salts in human performance and postmortem toxicology: method development, drug distribution and interpretation of results. J Anal Toxicol 37, 135-146.

Marona-Lewicka, D., Rhee, G.S., Sprague, J.E., Nichols, D.E., 1995. Psychostimulant-like effects of p-fluoroamphetamine in the rat. Eur J Pharmacol 287, 105-113.

Marusich, J.A., Antonazzo, K.R., Wiley, J.L., Blough, B.E., Partilla, J.S., Baumann, M.H., 2014. Pharmacology of novel synthetic stimulants structurally related to the "bath

salts" constituent 3,4-methylenedioxypyrovalerone (MDPV). Neuropharmacology, in press.

Meltzer, P.C., Butler, D., Deschamps, J.R., Madras, B.K., 2006. 1-(4-Methylphenyl)-2-pyrrolidin-1-yl-pentan-1-one (Pyrovalerone) analogues: a promising class of monoamine uptake inhibitors. J Med Chem 49, 1420-1432.

Nichols, D.E., 2004. Hallucinogens. Pharmacol Ther 101, 131-181.

Pei, Y., Lee, J., Leo, D., Gainetdinov, R.R., Hoener, M.C., Canales, J.J., 2014. Activation of the trace amine-associated receptor 1 prevents relapse to cocaine seeking. Neuropsychopharmacology, in press.

Revel, F.G., Moreau, J.L., Gainetdinov, R.R., Bradaia, A., Sotnikova, T.D., Mory, R., Durkin, S., Zbinden, K.G., Norcross, R., Meyer, C.A., Metzler, V., Chaboz, S., Ozmen, L., Trube, G., Pouzet, B., Bettler, B., Caron, M.G., Wettstein, J.G., Hoener, M.C., 2011. TAAR1 activation modulates monoaminergic neurotransmission, preventing hyperdopaminergic and hypoglutamatergic activity. Proc Natl Acad Sci U S A 108, 8485-8490.

Rohrich, J., Becker, J., Kaufmann, T., Zorntlein, S., Urban, R., 2012. Detection of the synthetic drug 4-fluoroamphetamine (4-FA) in serum and urine. Forensic Sci Int 215, 3-7.

Rust, K.Y., Baumgartner, M.R., Dally, A.M., Kraemer, T., 2012. Prevalence of new psychoactive substances: a retrospective study in hair. Drug Test Anal 4, 402-408.

Scholze, P., Zwach, J., Kattinger, A., Pifl, C., Singer, E.A., Sitte, H.H., 2000. Transporter-mediated release: a superfusion study on human embryonic kidney cells stably expressing the human serotonin transporter. J Pharmacol Exp Ther 293, 870-878.

Simmler, L., Buser, T., Donzelli, M., Schramm, Y., Dieu, L.H., Huwyler, J., Chaboz, S., Hoener, M., Liechti, M.E., 2013. Pharmacological characterization of designer cathinones in vitro. Br J Pharmacol 168, 458-470.

Simmler, L.D., Hysek, C.M., Liechti, M.E., 2011. Sex differences in the effects of MDMA (ecstasy) on plasma copeptin in healthy subjects. J Clin Endocrinol Metab 96, 2844-2850.

Simmler, L.D., Rickli, A., Hoener, M.C., Liechti, M.E., 2014a. Monoamine transporter and receptor interaction profiles of a new series of designer cathinones. Neuropharmacology 79, 152-160.

Simmler, L.D., Rickli, A., Schramm, Y., Hoener, M.C., Liechti, M.E., 2014b. Pharmacological profiles of aminoindanes, piperazines, and pipradrol derivatives. Biochem Pharmacol 88, 237-244.

Smollin, C., Murray, S., Gerona, R., 2011. An unexpected finding of MDPPP in a patient with severe agitation and hallucinations. Clin Toxicol (Phila) 49, 525-526.

Spiller, H.A., Ryan, M.L., Weston, R.G., Jansen, J., 2011. Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin Toxicol (Phila) 49, 499-505.

Tatsumi, M., Groshan, K., Blakely, R.D., Richelson, E., 1997. Pharmacological profile of antidepressants and related compounds at human monoamine transporters. Eur J Pharmacol 340, 249-258.

Thornton, S.L., Lo, J., Clark, R.F., Wu, A.H., Gerona, R.R., 2012. Simultaneous detection of multiple designer drugs in serum, urine, and CSF in a patient with prolonged psychosis. Clin Toxicol (Phila) 50, 1165-1168.

Watterson, L.R., Kufahl, P.R., Nemirovsky, N.E., Sewalia, K., Grabenauer, M., Thomas, B.F., Marusich, J.A., Wegner, S., Olive, M.F., 2014. Potent rewarding and reinforcing effects of the synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV). Addict Biol, 19, 165-174.

Wee, S., Anderson, K.G., Baumann, M.H., Rothman, R.B., Blough, B.E., Woolverton, W.L., 2005. Relationship between the serotonergic activity and reinforcing effects of a series of amphetamine analogs. J Pharmacol Exp Ther 313, 848-854.

Westphal, F., Junge, T., Klein, B., Fritschi, G., Girreser, U., 2011. Spectroscopic characterization of 3,4-methylenedioxypyrrolidinobutyrophenone: a new designer drug with alpha-pyrrolidinophenone structure. Forensic Sci Int 209, 126-132.

Winstock, A.R., Mitcheson, L.R., Deluca, P., Davey, Z., Corazza, O., Schifano, F., 2011. Mephedrone, new kid for the chop? Addiction 106, 154-161.

Zuba, D., Byrska, B., 2013. Prevalence and co-existence of active components of 'legal highs'. Drug Test Anal 5, 420-429.

Figure Legends

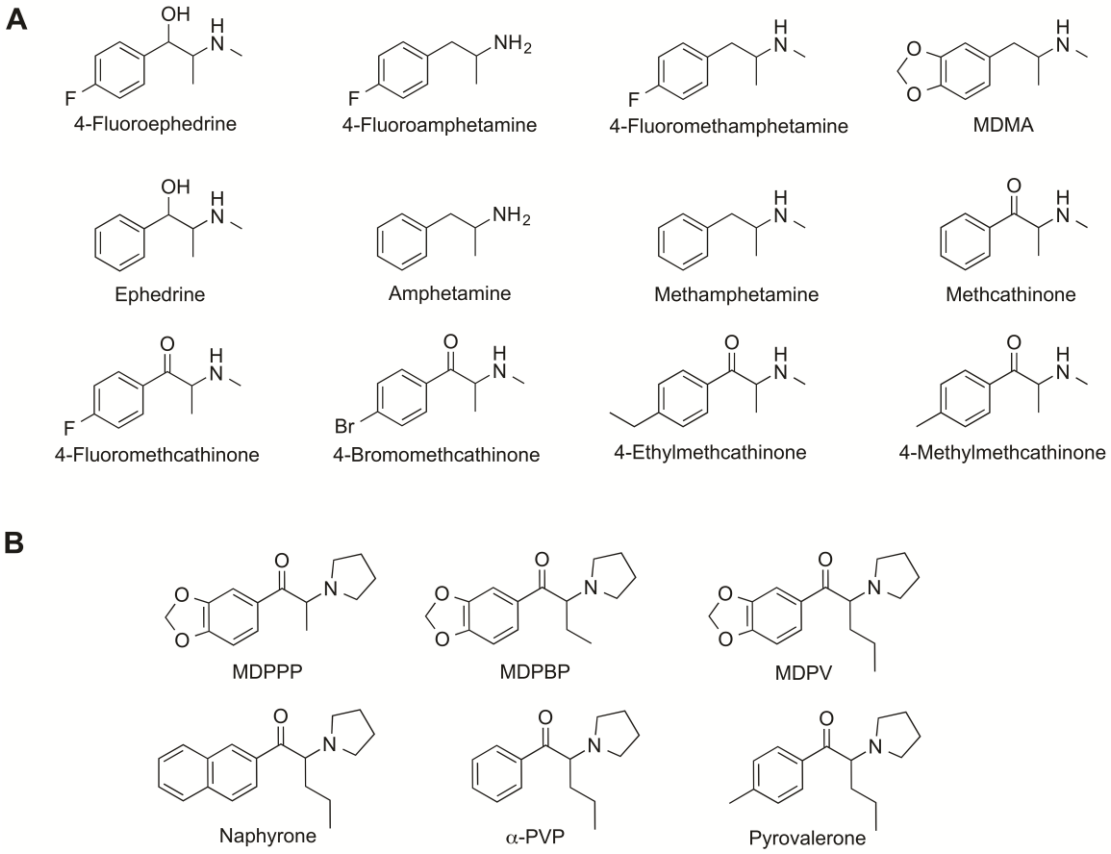


Figure 1. Chemical structures of novel psychoactive substances. **A.** Para-(4)-substituted amphetamines, 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"), and other classic non-para-substituted amphetamines. **B.** Pyrovalerone-type cathinones.

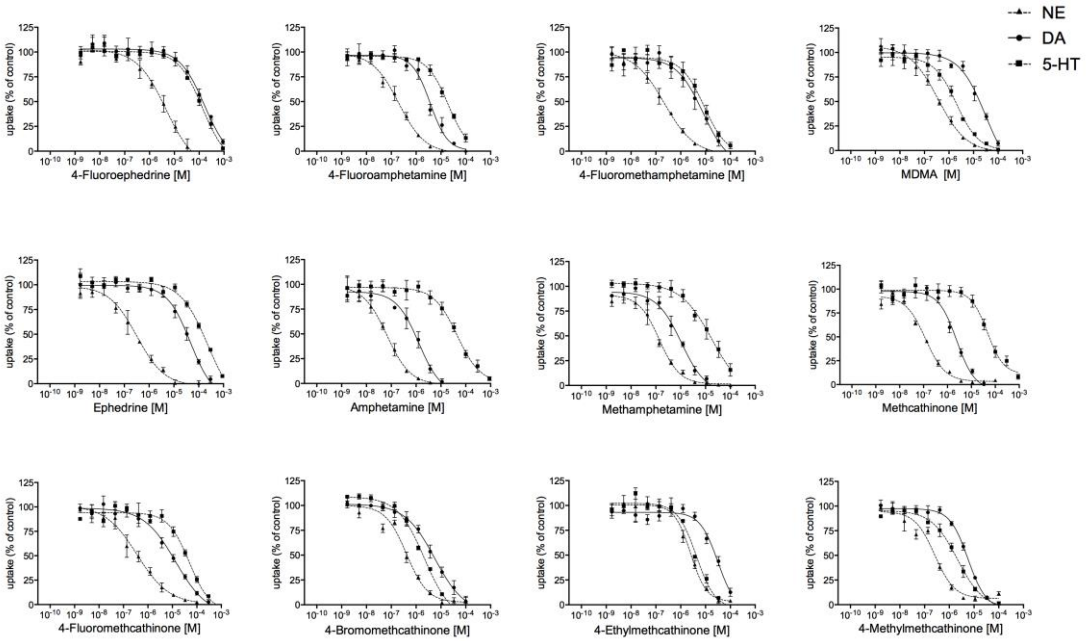


Figure 2. Effects of para-(4)-substituted and non-substituted amphetamines on monoamine transport. Monoamine uptake inhibition is presented as concentration-response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as the mean ± SEM of 3-4 independent experiments. The lines show the data fit by nonlinear regression. The corresponding IC_{50} values are shown in Table 1.

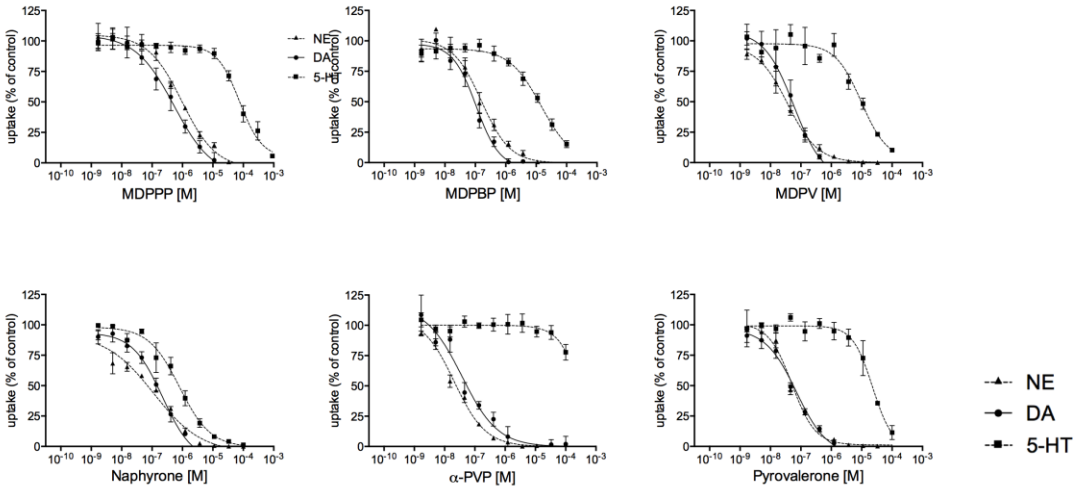
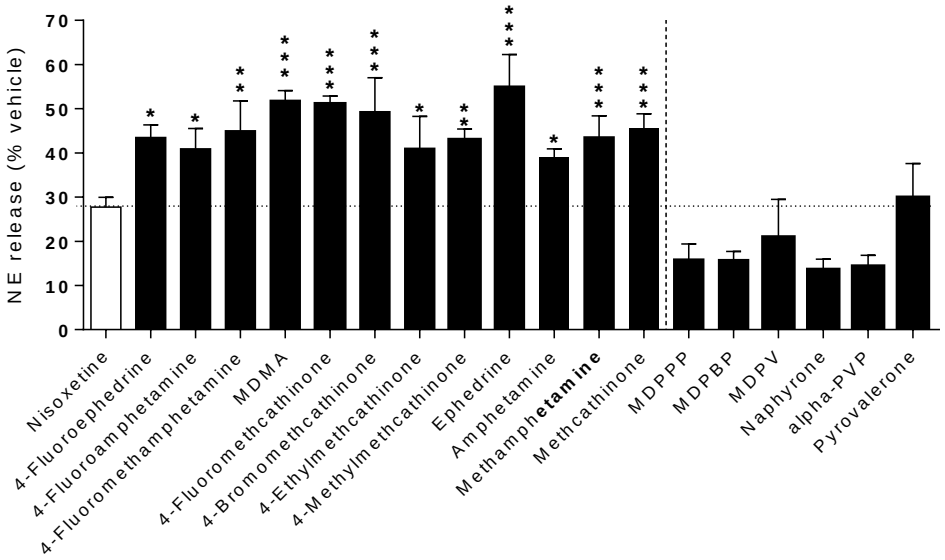
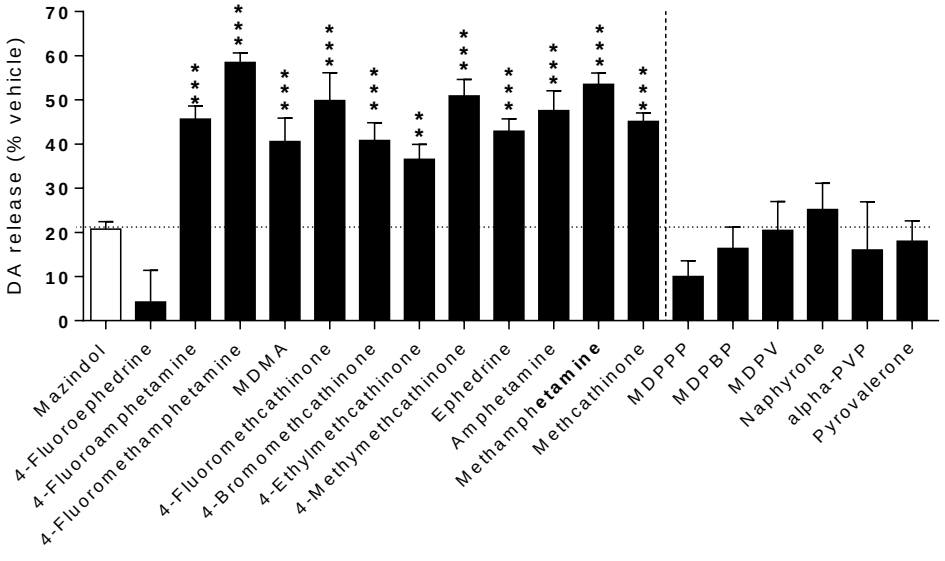


Figure 3. Effects of pyrovalerone cathinones on monoamine transport. Monoamine uptake inhibition is presented as concentration-response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as the mean ± SEM of 3-4 independent experiments. The lines show the data fit by nonlinear regression. The corresponding IC50 values are shown in Table 1.


NE release (% vehicle)
70
60
50
40
30
20
10
0
Nisoxetine
4-Fluoroephedrine
4-Fluoroamphetamine
4-Fluoromethamphetamine
MDMA
4-Fluoromethcathinone
4-Bromomethcathinone
4-Ethylmethcathinone
4-Methylmethcathinone
Ephedrine
Amphetamine
Methamphetamine
Methcathinone
MDPPP
MDPBP
MDPV
Naphyrone
alpha-PVP
Pyrovalerone


DA release (% vehicle)
70
60
50
40
30
20
10
0
Mazindol
4-Fluoroephedrine
4-Fluoroamphetamine
4-Fluoromethamphetamine
MDMA
4-Fluoromethcathinone
4-Bromomethcathinone
4-Ethylmethcathinone
4-Methylmethcathinone
Ephedrine
Amphetamine
Methamphetamine
Methcathinone
MDPPP
MDPBP
MDPV
Naphyrone
alpha-PVP
Pyrovalerone

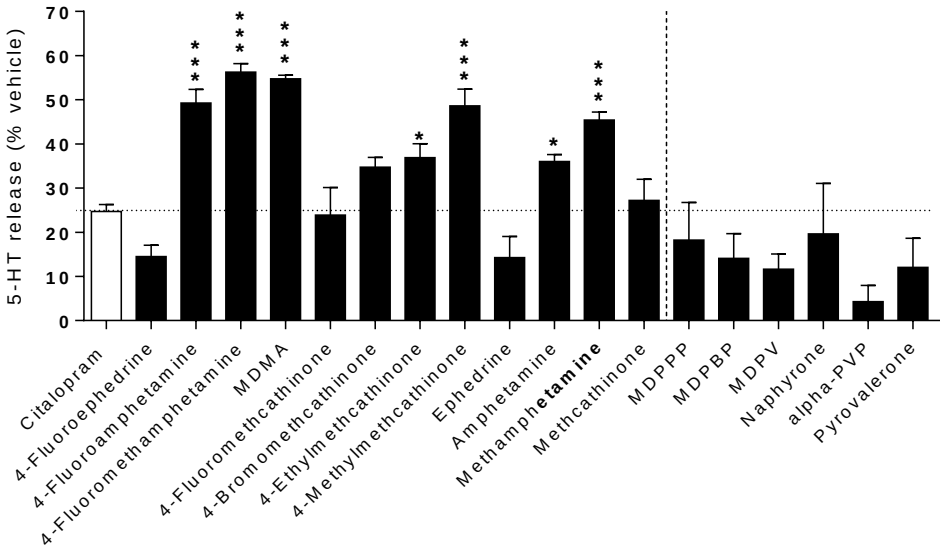

5-HT release (% vehicle)
70
60
50
40
30
20
10
0
Citalopram
4-Fluoroephedrine
4-Fluoroamphetamine
4-Fluoromethamphetamine
MDMA
4-Fluoromethcathinone
4-Bromomethcathinone
4-Ethylmethcathinone
4-Methylmethcathinone
Ephedrine
Amphetamine
Methamphetamine
Methcathinone
MDPPP
MDPBP
MDPV
Naphyrone
alpha-PVP
Pyrovalerone

Figure 4. Effect of all substances on monoamine release. HEK 293 cells that expressed NET, DAT, and SERT were loaded with [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT, respectively, washed, and incubated with a high concentration of the compounds (100 µM). All para-substituted and non-substituted amphetamines released NE, DA, or 5-HT (substances on the left of the vertical dashed line). In contrast, the pyrovalerone cathinones did not release monoamines (substances on the right of the vertical dashed line). Monoamine release is expressed as the percent reduction of monoamine cell content compared with vehicle (0% = no release; 100% release would indicate that all of the monoamine was released from the cells). Non-releasing monoamine transporter blockers induce nonspecific "pseudo-efflux" (horizontal dashed line, open bars), which arises from substrate that diffuses out of the cells and from subsequent reuptake inhibition. Compounds that produced significantly more monoamine efflux (*$p < 0.05$, *p<0.01, ***$p < 0.001$) compared with the non-releasing uptake inhibitors (negative controls, open bars) nisoxetine (HEK-NET cells), mazindol (HEK-DAT cells), and citalopram (HEK-SERT cells) were considered monoamine releasers. The data are expressed as the mean ± SEM of 3-4 independent experiments.

Table 1. Monoamine transporter inhibition.

	NET	DAT	SERT	DAT/SERT inhibition ratio
	IC50 [µM] (95% CI)	IC50 [µM] (95% CI)	IC50 [µM] (95% CI)	Ratio (95% CI)
Para-(4)-substituted amphetamines				
4-Fluoroephedrine	4.5 (2.0-11)	163 (40-668)	134 (76-236)	0.8 (0.1-5.9)
4-Fluoroamphetamine	0.20 (0.14-0.28)	3.7 (2.4-5.7)	19 (11-33)	5.1 (1.9-14)
4-Fluoromethamphetamine	0.22 (0.14-0.35)	7.7 (2.5-24)	8.7 (3.8-20)	1.1 (0.2-8.0)
MDMA	0.36 (0.23-0.57)	31 (8-118)	2.0 (1.4-3.0)	0.06 (0.01-0.4)
4-Fluoromethcathinone	0.36 (0.17-0.75)	14 (7.5-24)	49 (30-80)	3.6 (1.3-11)
4-Bromomethcathinone	0.41 (0.30-0.57)	5.6 (2.7-12)	2.2 (1.7-2.8)	0.4 (0.1-1.0)
4-Ethylmethcathinone	2.5 (1.7-3.7)	31 (13-72)	4.3 (3.2-5.9)	0.14 (0.04-0.5)
4-Methylmethcathinone	0.26 (0.17-0.39)	5.7 (4.3-7.5)	2.1 (1.6-2.7)	0.4 (0.2-0.7)
non para-(4)-substituted amphetamines				
Ephedrine	0.32 (0.21-0.50)	46 (27-79)	230 (72-735)	5.0 (0.9-27)
Amphetamine	0.07 (0.05-0.1)	1.3 (0.8-2.0)	45 (24-85)	35 (12-106)
Methamphetamine	0.14 (0.09-0.22)	1.1 (0.7-1.7)	18 (3-116)	17 (1.8-166)
Methcathinone	0.12 (0.09-0.15)	2.4 (1.7-3.4)	46 (30-71)	19 (8.8-42)
Pyrovalerone cathinones				
MDPPP	0.97 (0.62-1.5)	0.53 (0.27-1.1)	75 (49-114)	141 (45-422)
MDPBP	0.16 (0.11-0.24)	0.11 (0.07-0.16)	15 (5.4-39)	132 (34-557)
MDPV	0.04 (0.03-0.05)	0.05 (0.04-0.06)	9.6 (3.4-27)	192 (57-675)
Naphyrone	0.11 (0.05-0.27)	0.22 (0.16-0.31)	0.80 (0.6-1.2)	3.6 (1.9-7.5)
α-PVP	0.02 (0.01-0.03)	0.04 (0.01-0.1)	> 100	> 1000
Pyrovalerone	0.05 (0.04-0.07)	0.07 (0.05-0.11)	23 (9.7-54)	327 (88-1080)

Values are means of three to four independent experiments and 95% confidence intervals (CI). DAT/SERT inhibition ratio = 1/DAT IC_{50} : 1/SERT IC_{50}.

Table 2. Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	a_{1A}	a_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$	$TAAR_{1human}$
Para-(4)-substituted amphetamines												
4-Fluoroephedrine	17.6 ± 2	27.7 ± 15	39 ± 11	> 4.9	8.4 ± 1.2	> 12	> 20	> 17	> 13	2.6 ± 1.2	17.6 ± 8	NA
4-Fluoroamphetamine	13.5 ± 2	11.0 ± 4	32 ± 9	> 4.9	4.4 ± 0.3	> 12	> 20	> 17	> 13	0.08±0.04	0.3 ± 0.1	NA
4-Fluoromethamphetamine	9.0 ± 0.6	10.8 ± 1	35 ± 12	> 4.9	2.6 ± 0.3	> 12	> 20	> 17	7.1 ± 2	0.24 ± 0.1	1.7 ± 0.9	NA
MDMA*	26.8 ± 9	8.4 ± 3	13.0 ± 2	> 6	15.0 ± 10	> 12	25 ± 13	> 17	> 13	0.37 ± 0.1	2.4 ± 1	14.6 ± 2
4-Fluoromethcathinone[#]	> 25	12.2 ± 3	> 30	1.5 ± 0.1	> 20	> 12	> 30	> 17	> 13	5.4 ± 2	> 10	> 20
4-Bromomethcathinone	6.5 ± 1	3.6 ± 0.3	8.3 ± 2	8.2 ± 3	12.7 ± 0.2	> 12	>10	> 17	2.1 ± 0.1	1.8 ± 0.1	12.9 ± 3	NA
4-Ethylmethcathinone	16.2 ± 2	28 ± 16	17.5 ± 4	8.4 ± 3	21.1 ± 8	> 12	>10	> 17	> 13	> 20	> 20	NA
4-Methylmethcathinone[#]	> 25	3.4 ± 0.8	> 30	3.5 ± 2	11.0 ± 5	> 12	> 30	> 9	> 13	4.3 ± 2	> 10	> 20
Non para-(4)-substituted amphetamines												
Ephedrine	> 30	> 30	> 30	> 12	4.1 ± 0.5	> 12	> 25	> 17	> 13	3.7±0.9	> 14.6	NA
Amphetamine[#]	1.0 ± 0.6	5.7 ± 4	> 25	> 6	2.8 ± 0.8	> 12	> 30	> 17	> 13	0.23 ± 0.2	0.09 ± 0.06	0.22 ± 0.1
Methamphetamine*	3.0 ± 2	1.8 ± 0.7	24.6 ± 10	> 6	6.1 ± 2	> 12	> 30	> 17	> 13	0.35 ± 0.1	0.55 ± 0.2	1.4 ± 0.5
Methcathinone[#]	1.4 ± 0.7	1.3 ± 0.2	> 30	3.9 ± 1	11.9 ± 4	> 12	> 30	> 9	> 13	4.1 ± 1	> 10	> 20
Pyrovalerone cathinones												
MDPBP	1.1 ± 0.1	0.02 ± 0.002	4.1 ± 1	> 4.9	9.4 ± 2	> 12	> 20	> 17	> 13	> 20	> 20	NA
MDPPP	3.5 ± 1	0.18 ± 0.05	11.7 ± 1	> 15	13.9 ± 0.9	> 12	> 10	> 17	8.7 ± 0.6	16.1 ± 7	> 20	NA
MDPV[#]	0.08 ± 0.02	0.01 ± 0.002	2.9 ± 0.1	> 6	> 20	> 12	> 30	> 9	> 13	7.2 ± 1.1	> 10	> 20
Naphyrone[#]	0.18 ± 0.02	0.04 ± 0.01	0.18 ± 0.02	> 6	7.9 ± 3	> 12	> 20	> 17	2.3 ± 0.3	> 20	> 20	> 20
α-PVP	0.06 ± 0.02	0.007 ± 0.002	> 30	> 15	31.7 ± 2	> 12	> 10	> 17	> 13	16.3 ± 6	> 20	NA
Pyrovalerone[#]	0.06 ± 0.01	0.03 ± 0.005	5.0 ± 0.3	> 6	> 20	> 12	> 30	> 9	10.7 ± 2	> 12	> 10	> 20

Values are Ki given as microM (mean ±SD); NA, not assessed
*values are from Simmler et al. 2013 except for the TAAR1human binding
#values are from Simmler et al. 2014 except for the TAAR1human binding

Table 3. Serotonin5-HT$_{2A}$ and 5-HT$_{2C}$ receptor binding affinities and functional 5-HT$_{2B}$ receptor activity

	5-HT$_{1A}$	5-HT$_{2A}$	5-HT$_{2B}$		5-HT$_{2C}$
	receptor binding Ki ± SEM (μM)	receptor binding Ki ± SEM (μM)	activation potency EC_{50} ± SEM (μM)	activation efficacy % maximum ± SEM	receptor binding Ki ± SEM (μM)
Para-(4)-substituted amphetamines					
4-Fluoroephedrine	> 17	> 12.5	> 20	0	3.7 ± 1.1
4-Fluoroamphetamine	4.4 ± 0.8	11.3 ± 3	14.4 ± 7	58 ± 20	7.8 ± 0.7
4-Fluoromethamphetamine	5.0 ± 2	3.8 ± 0.7	> 20	0	9.5 ± 5.0
MDMA*	12.2 ± 1	5.9 ± 3	> 20	0	> 13
4-Fluoromethcathinone#	> 20	1.4 ± 0.6	> 20	0	> 13
4-Bromomethcathinone	> 20	3.3 ± 0.6	> 20	0	>13
4-Ethylmethcathinone	> 20	6.5 ± 0.9	> 20	0	9.6 ± 0.4
4-Methylmethcathinone#	> 20	2.1 ± 0.7	> 20	0	> 13
Non para-(4)-substituted amphetamines					
Ephedrine	> 20	> 13	> 20	0	3.3 ± 0.7
Amphetamine#	6.7 ± 1	> 13	9.7 ± 2.2	9 ± 2	> 13
Methamphetamine*	8.1 ± 1	> 13	> 20	0	> 13
Methcathinone#	12.8 ± 4	3.0 ± 0.6	> 20	0	> 13
Pyrovalerone cathinones					
MDPBP	13.0 ± 0.02	> 13	> 20	0	> 13
MDPPP	2.5 ± 0.3	7.5 ± 0.1	> 20	0	> 13
MDPV#	10.3 ± 5	> 13	> 20	0	> 13
Naphyrone#	6.0 ± 0.2	11.7 ± 2	>20	0	> 13
α-PVP	5.2 ± 0.1	> 13	> 20	0	> 13
Pyrovalerone#	13.4± 2	> 13	> 20	0	> 13

NA, not assessed; binding values are from *Simmler et al. 2013 or #Simmler et al. 2014, respectively and are included for comparison.