Pharmacology of novel psychoactive substances

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"When you study natural science and the miracles of creation, if you don't turn into a mystic you are not a natural scientist."

Albert Hofmann

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Abbreviations

2-AI	2-Aminoindane
2-DPMP	Desoxypipradrol
2C-B-FLY	8-Bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine
2C-P	2-(2,5-dimethoxy-4-propylphenyl)ethanamine
3-FMC	3-Fluoromethcathinone
4-APB	4-(2-Aminopropyl)benzofuran
4-BMC	4-Bromomethcathinone
4-EMC	4-Ethylmethcathinone
4-FA	4-Fluoroamphetamine
4-FEP	4-Fluoroephedrine
4-FMA	4-Fluoromethamphetamine
4-FMC	4-Fluoromethcathinone
4-MEC	4-Methylethcathinone
4-MMC	4-Methylmethcathinone
4-MTA	4-Methylthioamphetamine
5-APB	5-(2-aminopropyl)benzofuran
5-APDB	5-(2-Aminopropyl)-2,3-dihydrobenzofuran
5-HT	5-Hydroxytryptamine, serotonin
5-IAI	5-Iodo-2-aminoindane
5-MAPDB	1-(2,3-dihydrobenzofuran-5-yl)-N-methylpropan-2-amine
6-APB	6-(2-aminopropyl)benzofuran
6-APDB	6-(2-Aminopropyl)-2,3-dihydrobenzofuran
7-APB	7-(2-Aminopropyl)benzofuran
alpha-PVP	α-Pyrrolidinopentiophenone
bk-MDA	β-keto-3,4-Methylenedioxyamphetamine, 3,4-Methylenedioxycathinone
Buphedrone	α-methylamino-Butyrophenone
BZP	Benzylpiperazine
D2PM	Diphenylprolinol
DA	Dopamine
DAT	Dopamine transporter
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
LSD	Lysergic acid diethylamide
m-CPP	meta-Chlorophenylpiperazine
MAO	Monoamine oxidase
MDA	3,4-Methylenedioxyamphetamine
MDAI	5,6-Methylenedioxy-2-aminoindane
MDMA	3,4-Methylenedioxymethamphetamine
MDPBP	3’,4’-Methylenedioxy-α-pyrrolidinobutiophenone
MDPPP	3’,4’-Methylenedioxy-α-pyrrolidinopropiophenone
MDPV	3,4-Methylenedioxypyrovalerone
Methedrone	4-Methoxymethcathinone
MPH	Methylphenidate

N,N-DMC	N,N-Dimethylcathinone
NBOMe	N-(2-methoxy)benzyl
NE	Norepinephrine
NET	Norepinephrine transporter
NPS	Novel psychoactive substance
Pentedrone	α-methylamino-Valerophenone
Pentylone	β-keto-Methylbenzodioxolylpentanamine
PMA	4-Methoxyamphetamine
PMMA	4-Methoxymethamphetamine
SERT	Serotonin transporter
$TAAR_1$	Trace amine-associated receptor 1
TFMPP	Trifluoromethylphenylpiperazine
$VMAT_2$	Vesicular monoamine transporter 2

Summary

This PhD work consists of an *in vitro* and *in vivo* part. In the *in vivo* part, we investigated the role of dopamine in the acute clinical effects of 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) in healthy human subjects. The role of dopamine in the addictive effects of drug of abuse is well established, but whether it contributes to the acute psychotropic effects of MDMA is unclear.

In this pharmacological interaction study, we used the dopamine and weak norepinephrine transporter inhibitor bupropion (Stahl et al. 2004) as a pharmacological tool to block the MDMA-induced dopamine release and to study the role of dopamine in the effects of MDMA. We hypothesized that bupropion would decrease the subjective effects of MDMA to the extent that they depend on MDMA-induced release of dopamine.

We included 16 healthy human subjects in this double-blind, placebo-controlled, crossover study. Bupropion pretreatment slightly increased MDMA plasma concentration and prolonged but not reduced the subjective effects contrary to our hypothesis. Additionally, bupropion reduced the MDMA-induced elevations in plasma norepinephrine concentrations and the heart rate response to MDMA.

These findings support a role for norepinephrine in the MDMA-induced cardiostimulant effects but no role for MDMA-induced transporter-mediated dopamine release in the elevated mood effects after MDMA administration. Possibly, most of the acute psychotropic effects of MDMA are mediated via transporter-mediated release of serotonin and norepinephrine as previously shown (Hysek et al. 2011, Hysek et al. 2012).

In the second and main part of this work we characterized the pharmacological profiles of novel psychoactive substances (NPS). Specifically, we studied whether and how potently NPS interacted with the human transporters for norepinephrine, dopamine, and serotonin, stably expressed in human embryonic kidney (HEK293) cells. Additionally, we assessed binding affinity to the serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}-receptors and the activation potency and activation efficacy at 5-HT_{2A} and 5-HT_{2B} receptors. Furthermore, binding to alpha$_{1A/2A}$-adrenergic, dopamine D_{1-3}, histamine H_1 receptors, as well as trace amine-associated receptor 1 ($TAAR_1$) was also assessed.

The NPS studied in this project included para-4-halogenated amphetamine derivatives, which were shown to be relatively more serotonergic than their non-4-halogenated counterparts and pyrovaleronering-substituted cathinones, which were highly potent dopamine transporter inhibitors with a high risk for abuse.
Para-halogenated drugs (4-fluoroephedrine, 4-fluoroamphetamine, 4-fluoromethamphetamine, 4-fluoromethcathinone, and 4-bromomethcathinone) also released monoamines, similar to MDMA, whereas pyrovalerones were found to be pure uptake inhibitors. Most benzofurans were similar to MDMA but slightly more serotoninergic than MDMA and additionally activated the serotonin 5-HT_{2B} receptor.
The last big group of NPS studied in this project, were novel hallucinogens, which predominantly interacted with the 5-HT_{2A} receptor. This serotonin receptor subtype mediates the hallucinogenic and hallucinogenic-like visual effects of classic serotonergic hallucinogens (Vollenweider et al. 1998, Nichols 2004, Halberstadt et al. 2013, Halberstadt et al. 2014, Halberstadt 2015).
Compounds tested in this project included the benzodifuran 8-Bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine (2C-B-FLY), 2C-drugs with their highly potent N-(2-methoxy)benzyl (NBOMe)-derivatives, and lysergic acid diethylamide (LSD). Interestingly, NBOMe derivatives displayed higher affinities at the 5-HT_{2A} receptor than LSD, together with a high selectivity for 5-HT_{2A} over the 5-HT_{1A} receptor, contrary to LSD. NBOMes were partial 5-HT_{2A} receptor agonists, similar to LSD. These novel drugs likely carry a high hallucinogenic potential when used recreationally by humans and the high binding to α_{1A}-receptor (K_i < 1µM) may result in additional vasocontrictive and cardiovascular stimulant effects.

Taken together, this PhD contributed to the understanding of the role of dopamine in the effects of MDMA, an important recreational substances. Additionally, we characterized the *in vitro* pharmacology of many novel designer drugs, which will be helpful in the prediction of the clinical toxicological effects of these newly used recreational drugs.

Introduction

Overview: Classification and relevance

The today drug market is volatile and especially the Internet serves as an ideal tool to obtain any kind of psychoactive substance. NPS are sold as “bath salts”, “plant food” or “research chemicals” and labelled “not for human consumption” to circumvent legislation and mimic psychoactive effects of banned classical drugs including MDMA, methamphetamine, cocaine, or LSD. The European Monitoring Center for Drugs and Drug Addiction (EMCDDA) defines NPS as follows:

“A new psychoactive substance is defined as a new narcotic or psychotropic drug, in pure form or in preparation, that is not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions” (Iversen 2015)

Statistics of the EMCDDA show clearly an ongoing increase in the number of NPS detected in the EU. At least 400 NPS have been reported in the last few years and in 2014, with 101 NPS noted by the EMCDDA, presented in Figure 1 (EMCDDA 2015, Wood et al. 2015).

Most newly detected designer drugs belong to the phenethylamines and synthetic cathinones or to the structurally diverse group of synthetic cannabinoids. Although NPS are not responsible for as many deaths as alcohol, benzodiazepines, prescribed opioids, cocaine, or heroin (Hansen et al. 2014, Martins et al. 2015, Nichols et al. 2015, Simonsen et al. 2015) they are a big health concern due to their unknown potential harm and their unknown pharmacological profile. Importantly, chemical substitutions may keep the effects of a controlled drug and lead to a legal alternative or in contrast, the chemical modification may result in totally different pharmacological and related toxicological effects. This makes it dangerous to consume NPS, since numerous NPS were involved in toxic effects and deaths alone or contributed to these effects in mixed-drug intoxications (Simmler et al. 2014, Dines et al. 2015, McAuley et al. 2015, Rickli et al. 2015, Rickli et al. 2015). Therefore the pharmacologic characterization of NPS is very important to assess their potential harm.

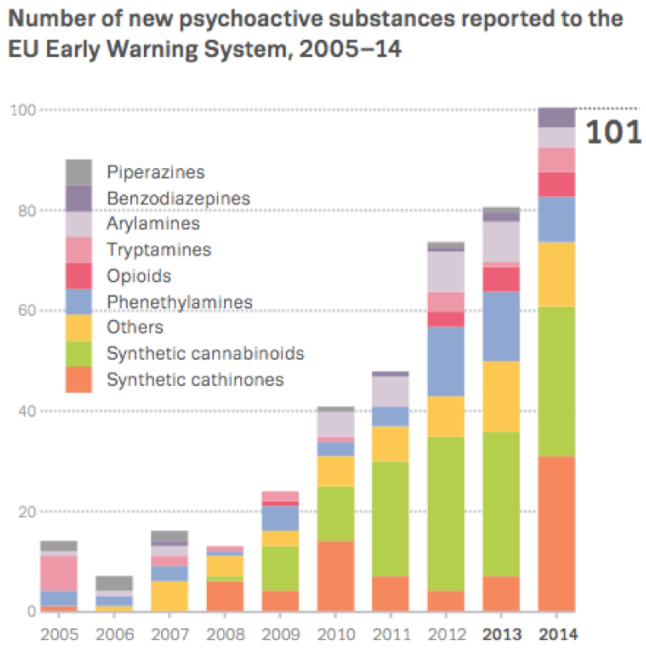


Figure 1: Number of new psychoactive substances reported to the EU Early Warning System, 2005-2014 (EMCDDA 2015).

Mechanism of action

Monoamine uptake inhibition

Amphetamine, MDMA, and many NPS interact with the norepinephrine (NE), dopamine (DA), and serotonin (5-HT) transporter (NET, DAT, and SERT, respectively). These plasma membrane monoamine transporters regulate the homeostasis of NE, DA, and 5-HT in the brain and are located in the peri-synaptic area, mostly expressed on the respective neurons (Torres et al. 2003). They terminate the signal of NE, DA, and 5-HT by reuptake of the transmitter into the synapse. The driven transport force is the ion gradient over the synaptic membrane maintained by the Na^+/K^+-ATPase (transporting potassium into the intracellular space and sodium out of the cytosol) (Sitte et al. 2015). Subsequent monoamine storage in the vesicles happens via the vesicular monoamine transporter 2 ($VMAT_2$) (Fleckenstein et al. 2003). Designer drugs with monoamine substrate properties, like MDMA or methamphetamine, are also transported into the cell via the transporter and release the respective monoamine via the transporter into the synaptic cleft. Additionally, the drugs may also interfere with the $VMAT_2$ and disturb the transmitter balance leading to a monoamine increase in the synaptic cytosol (Partilla et al. 2006). Monoamine oxidase A and B (MAO A and B) inhibition by certain

amphetamine derivatives may further enhance their own concentration and potentiate the effect of the monoamine concentrations in the intra-and extracellular space, by inhibiting the degradation of monoamines (Leonardi et al. 1994, Scorza et al. 1997). In contrast to the amphetamines, we found some NPS such as the pyrovalerone-type cathinones to be pure and very potent monoamine uptake inhibitors unlike other amphetamines. Possibly the high inhibition potency of these compounds at the DAT and NET explains their psychotropic properties in humans.

In our studies, we investigated *in vitro* interaction of NPS with the NET, DAT, and SERT. Some NPS were either weak or inactive at monoamine transporters (like hallucinogenic drugs including the 2C series, NBOMes, LSD) or they inhibited at least one monoamine reuptake transporter. Figure 2 shows schematically the potential sites of interaction of NPS with the NET, DAT, and SERT as well as other targets tested in *in vitro* studies.

Monoamine release

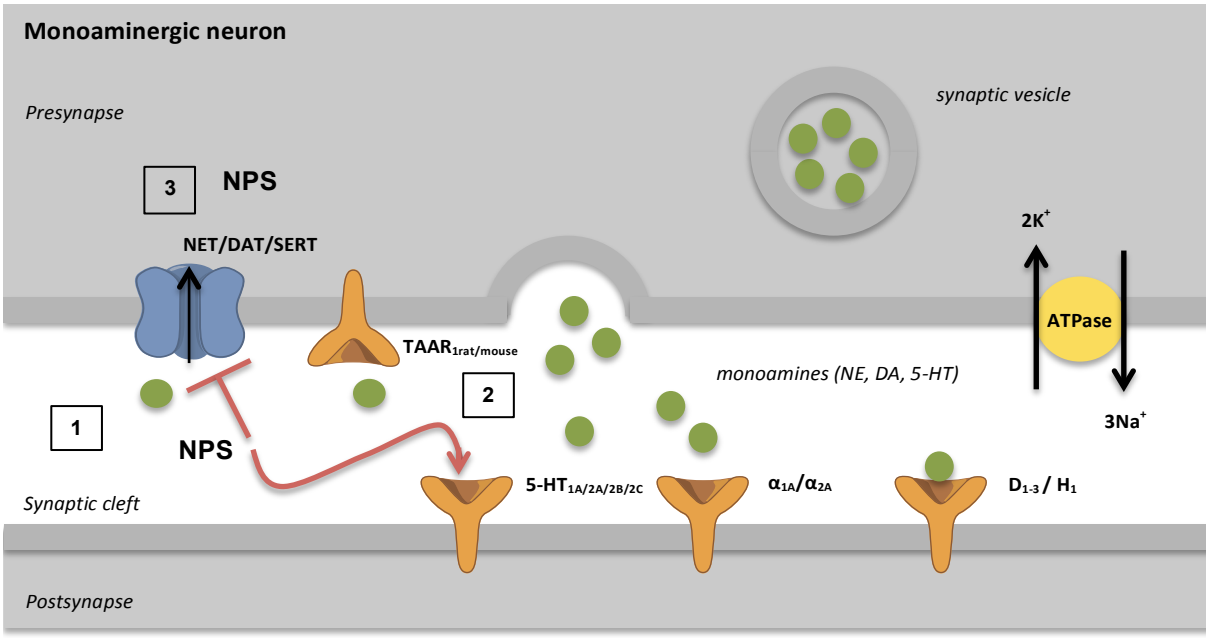
An increase of monoamines in the synaptic cleft can either happen via vesicular release or inhibition of the reuptake transporters. Amphetamine mediates its biologic response primarily via transporter-mediated neurotransmitter release mediated by the uptake transporters. Also MDMA and other compounds are taken up as substrates by these transporters (Eshleman et al. 2013, Simmler et al. 2013, Sitte et al. 2015).

The exact mechanism, by which amphetamine and other substrates induce monoamine release is not fully understood. However, there are several theories for this phenomenon. One model describes the uptake of a substrate and subsequent monoamine release via alternating access hypothesis of substrate translocation at the monoamine transporters (Manepalli et al. 2012, Sitte et al. 2015).

In this model, the carrier presents a pocket where substrate and co-transported sodium (Na^+) and chloride (Cl^-) can bind. With this co-transport as driving force, the transporter protein conformation changes and switch from the outwardfacing to the inwardfacing state and releases the substrate in the cytosol (Jones et al. 1999). The following change in the sodium gradient has been proposed as a factor for the subsequent induction of neurotransmitter release, since inhibition of Na^+/K^+-ATPase with ouabain increased monoamine efflux (Scholze et al. 2000). Thus this reduced sodium gradient can trigger a conformational change of the transporter and induce

monoamine release together in the presence of amphetamine and other substrates (Sitte et al. 2010). A second model considers the monoamine transporters working in a channel mode (Adam SV 2002). This model was proposed because observations indicated higher transporter-associated currents than the alternative access model could account for based on its stoichiometry. Therefore, charge was proposed to translocate through a channel-like transporter-state (Sonders et al. 1997). However, Schicker et al. (2012) found in human SERT, that uncoupled current is presented by a transiently formed state. Additionally, this state is in equilibrium with an inward facing and K^+-bound SERT mode. Therefore the extent of the channel-like state is limited and probably not the preferred model to describe the release-mechanism (Schicker et al. 2012).

A third hypothesis states an oligomer-based counter-transport model. It assumes that amphetamine or another substrate is taken up by one moiety of the transporter, which subsequently induces monoamine release through the other transporter. This model does not specify, which of the transporter account for the uptake of substrates and which one for the monoamine-release. Additionally, increasing extracellular substrate amount will probably reduce the release capacity, by occupancy of both oligomer-parts (Sitte et al. 2010).



NET: norepinephrine transporter; DAT: dopamine transporter; SERT: serotonin transporter; NPS: novel psychoactive substance; NE: norepinephrine; DA: dopamine; 5-HT: serotonin; TAAR: trace amine-associated receptor

1	Monoamine uptake inhibition by NPS
2	NPS interaction with monoamine receptors and transporters
3	NPS as monoamine substrates and following transporter reversal and monoamine release

Figure 2: Schematical presentation of interaction-sites of novel psychoactive substances with transporters and receptors *in vitro*.

1) Inhibition of NET, DAT, and/or SERT as a main site of action for many amphetamine-like drugs. Reduction of the monoamine clearance and recycling from the synaptic cleft, results in enhancement of the neurotransmitter-mediated signaling at the postsynaptic receptors. Monoamine reuptake transporters are driven by the potential gradient over the membrane, produced by the N^+/K^+-ATPase and co-transport of sodium and chloride.
2) Many NPS directly bind to presynaptic $TAAR_{1rat/mouse}$ and also postsynaptic serotonin, dopamine, and histamine receptors are possible interaction sites.
3) In addition to monoamine-reuptake inhibition described in 1), several reuptake-inhibitors serve also as a monoamine substrate either for NET, DAT, or/and SERT leading to a reversal of the monoamine transporter resulting in release of monoamines into the synaptic cleft.

Serotonin receptor interactions

The wide diversity of serotonin receptors include 7 different families with 14 serotonin (5-HT) receptors subtypes (Celada et al. 2013). They are G-protein coupled receptors (GPCR). For our studies we were interested in the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor subtypes. Serotonin receptors are involved in cognition, mood, anxiety, psychosis, sleep, schizophrenia, temperature regulation, appetite, sexual behavior, blood platelet aggregation, and muscle contraction (Cox 1977, Adams et al. 2002, Abbas et al. 2008, Przyklenk et al. 2010, Smith et al. 2010, Stein et al. 2015).

Serotonin 5-HT_{1A} receptors are located postsynaptically, but also on the presynaptic membrane and are hereby involved in the negative feedback mechanism induced by an increasing amount of serotonin in the synaptic cleft (Barnes et al. 1999, Celada et al. 2004). This subtype is broadly found in the CNS and ergolines like LSD bind to this receptor but not the hallucinogenic phenethylamines (Nichols 2004, Rickli et al. 2015).

The serotonin 5-HT_{2A} receptor has been considered as main target important for hallucinogenic effects produces by psychedelics with possible modulatory involvement of 5-HT_{1A} and 5-HT_{2C} receptors (Nichols 2004). The problem with the elucidation of the 5-HT_{2C} involvement is, that the 2A and 2C subtype share around 80% transmembrane (Cordova-Sintjago et al. 2012) structure homologies which challenges the development of a specific 5-HT_{2C} antagonist. Never the less, studies with the selective 5-HT_{2A} antagonist ketanserin in humans and M100’907 (Volinanserin) in animals showed the important role of 5-HT_{2A} receptors in the *in vivo* effects of hallucinogens (Vollenweider et al. 1998, Halberstadt et al. 2014).

Interactions with other receptors

NPS may also produce some of their effects by binding to monoamine-receptors including alpha$_{1A}$, alpha$_{2A}$, dopamine D_{1-3}, histamine receptor H_1, and trace amine-associated receptor 1 ($TAAR_1$).

For example, NPS may produce an increase in sympathetic activation via adrenergic alpha$_1$-receptors (Piascik et al. 2001). Alpha$_{1A\&2A}$ receptors belong to the big family of G-protein coupled receptors. The alpha$_{1A}$ receptor subtype is involved in the physiological responses to norepinephrine and epinephrine in the cardiovascular system (Chen et al. 2005). Alpha$_{2A}$ receptors are found throughout the CNS and also in the periphery in platelets, the spleen, kidney, eye, blood vessels, ileum, and adipocytes (Saunders et al. 1999). Alpha$_{1A}$ activation increases smooth muscle contraction, whereas alpha$_{2A}$ receptor agonists lower the vascular resistance and thereby the blood pressure by a negative feedback with inhibition of NE release in the brain.

Dopaminergic G-protein coupled receptors are broadly found in the brain and in peripheral tissues. Dopaminergic receptors regulate locomotion control, affect, emotion, and neuroendocrine secretion (Jaber et al. 1996). There are five dopamine subtypes, of which we included the D_1, D_2, and D_3 receptor in our studies. The dopamine receptor D_2 is possibly the most important subtype involved in learning, memory, psychosis, prolactin secretion, aldosterone secretion, regulation of sympathetic tone, regulation of renal function, blood pressure, vasodilatation, and gastro-intestinal tract motility. The dopamine system is also involved in several diseases such as Parkinson's disease or attention deficit hyperactivity disorder (ADHD) and D_2-antagonists are used in the treatment of schizophrenia. Dopamine D_1 and D_3 receptors are additionally important in locomotor activity, reward, and reinforcement mechanisms (Heidbreder et al. 2010, Beaulieu et al. 2011).

The histamine H1 receptor belongs also to the G-protein coupled receptors and is an important player in smooth muscle contraction, increase of vascular permeability, stimulation of hormone release, reduction of the heart contractibility, production of nitric oxide, and increase of neuronal firing (Hill et al. 1997). This receptor it is an important target mediating sedation, but our *in vitro* binding results suggest no involvement of the H_1 receptor in the effects of tested NPS. In contrast, the G-protein-linked $TAAR_1$ was found to modulate neurochemical and behavioral effects mediated by MDMA, methamphetamine, and cocaine, *in vitro* and in animals. These

studies found modulatory effects on dopaminergic and additional serotonergic circuits. $TAAR_1$ is mostly expressed intracellularly throughout the brain, especially in dopaminergic and adrenergic brain nuclei, and also found in the peripheral nervous system (Wolinsky et al. 2007, Miller 2011). Most NPS interacted with $TAAR_1$ and activation of this target may modulate the addictive and acute effects of these NPS as similarly described for the classic stimulants (Di Cara et al. 2011, Pei et al. 2014, Cotter et al. 2015).

Translation to clinic: Subjective effects and adverse reactions

Since there are still newly created designer drugs flooding the market and tested in uncontrolled recreational settings, the risk for overdosing and drug associated toxicity and adverse effects is high. Therefore, staying up to date regarding the pharmacology of these compounds is eminent, foremost for toxicologists and emergency physicians to aid in choosing the most appropriate treatment in case of intoxications. Although, *in vitro* data can only partly predict *in vivo* toxicity, parameters like the DAT/SERT inhibition ratio, presented in Figure 3, help to estimate whether a NPS has serotonergic MDMA-like effects (increased empathy for others, low addictive properties, low psychostimulation, risk of hyperthermia) associated with a low DAT/SERT inhibition ratio or rather methamphetamine-type stimulant effects (stimulation, high risk for addiction) associated with a high DAT/SERT inhibition ratio.

In addition to the receptor profile, pharmacokinetic properties such as absorption, route of administration, bioavailability, metabolism, and other factors contribute to the drug effects *in vivo*. Nevertheless, beside *in silico* binding studies (Reid et al. 2013), *in vitro* screenings are so far the first and simplest methods to pharmacologically characterize a novel compound and estimate its effects *in vivo*.

In summary, this thesis presents *in vitro* receptor and transporter interaction profiles of several groups of NPS that help to predict the toxicity of these substances in humans.

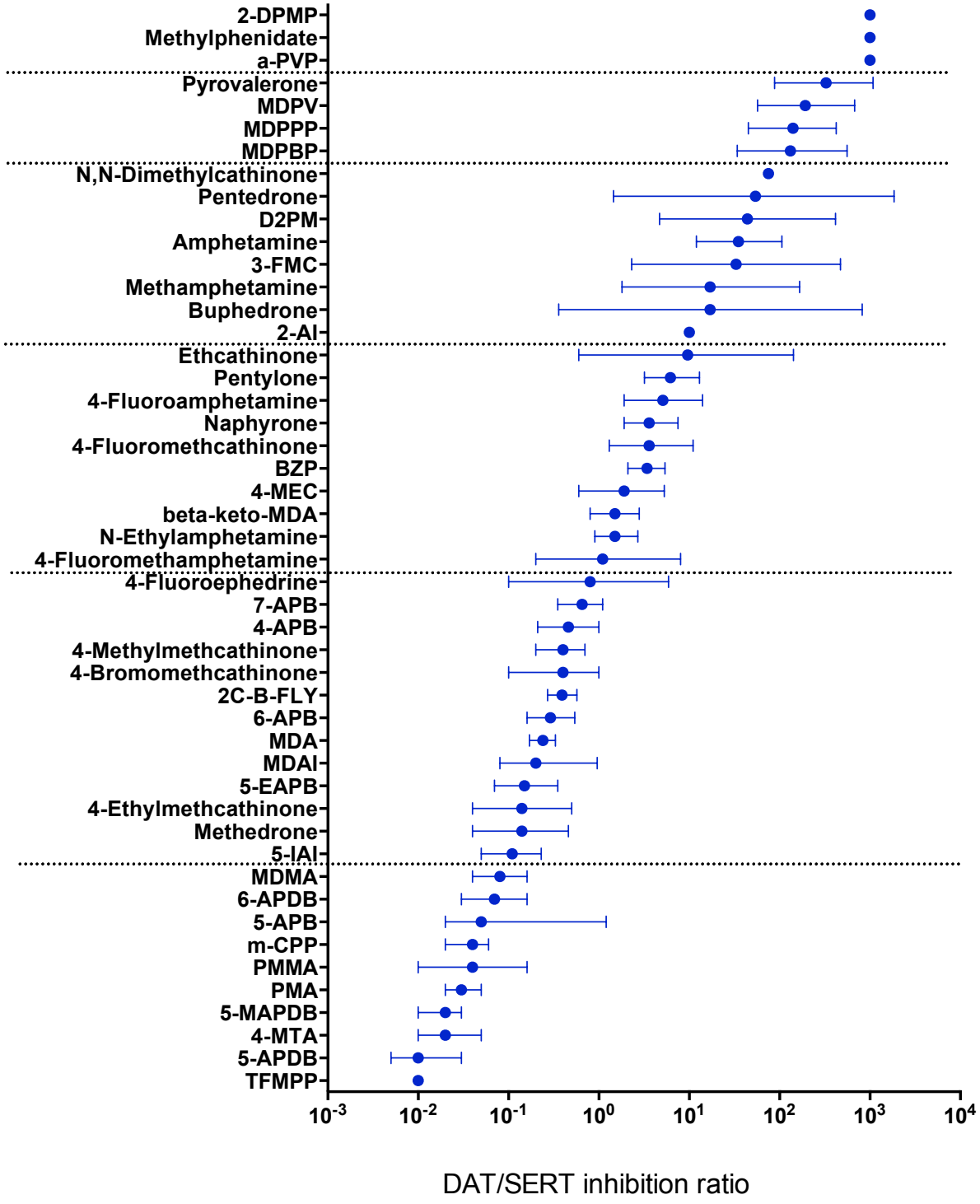


Figure 3: Ranking of the DAT/SERT inhibition ratio (1/DAT IC_{50} : 1/SERT IC_{50}) calculated for monoamine uptake inhibitors.

Values are means of three to five independent experiments and 95% confidence intervals (CI). A low DAT/SERT inhibition ratio indicates relative more serotonergic effects, whereas a high ratio predicts preliminary dopaminergic properties. Values without 95% CI may have an even lower (TFMPP), very wide (2-AI) or higher (N,N-Dimethylcatinone, α-PVP, methylphenidate, 2-DPMP) DAT/SERT inhibition ratio than indicated in this figure. Dotted lines separate the drugs in the following DAT/SERT ratio ranges: < 0.1: TFMPP - MDMA; 0.1-1.0: 5-IAI - 4-Fluoroephedrine; 1.0-10: 4-Fluoromethamphetamine - Ethcathinone; 10-100: 2-AI - N,N-Dimethylcathinone; 100-500: MDPBP - Pyrovalerone; > 500: α-PVP, methylphenidate, 2-DPMP

Objectives

Study questions

The aim of this PhD thesis was to get a better insight of the interaction of new psychoactive substances with monoamine transporters and a set of serotonergic and adrenergic receptors *in vitro*. Additionally, we assessed the role of the neurotransmitter dopamine in the acute affects of MDMA using the dopamine transporter inhibitor bupropion in an interaction study with MDMA in a controlled clinical trial in healthy human subjects.

Publications

The following section presents the peer-reviewed and published publications that form this PhD work, starting with the bupropion-MDMA-interaction study in healthy human subjects (paper 1). Then we present the monoamine transporter and receptor interactions profiles of designer cathinones, including also substituted amphetamines such as PMA, PMMA, pentylone, and others (paper 2), followed by a characterization of aminoindanes, piperazines, and pipradrol derivatives (paper 3). We then present data on MDMA-like para-halogenated NPS together with a group of potent catecholaminergic pyrovalerone-like (paper 4). Further, novel benzomonofurans served as MDMA replacement and this group of substances is discussed in paper 5 together with the potent hallucinogenic substances and benzodifuran 2C-B-FLY (paper 5). Finally, paper 6 is dedicated to the hallucinogenic 2C-drugs and their new NBOMe analogues. LSD and mescaline are classic serotonergic hallucinogens included in this study as comparators.

Paper 1

Interactions between bupropion and 3,4-methylenedioxymethamphetamine in healthy subjects.

Schmid Y, **Rickli A**, Schaffner A, Duthaler U, Grouzmann E, Hysek CM, Liechti ME.
J Pharmacol Exp Ther. 2015; 353(1): 102-11

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Interactions between Bupropion and 3,4-Methylenedioxymethamphetamine in Healthy Subjects

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA; "ecstasy") is a popular recreational drug. The aim of the present study was to explore the role of dopamine in the psychotropic effects of MDMA using bupropion to inhibit the dopamine and norepinephrine transporters through which MDMA releases dopamine and norepinephrine. The pharmacodynamic and pharmacokinetic interactions between bupropion and MDMA in 16 healthy subjects were investigated using a double-blind, placebo-controlled, crossover design. Bupropion reduced the MDMA-induced elevations in plasma norepinephrine concentrations and the heart rate response to MDMA. In contrast, bupropion increased plasma MDMA concentrations and prolonged its subjective effects. Conversely, MDMA increased plasma bupropion concentrations. These results indicate a role for the transporter-mediated release of norepinephrine in the cardiostimulant effects of MDMA but do not support a modulatory role for dopamine in the mood effects of MDMA. These results also indicate that the use of MDMA during therapy with bupropion may result in higher plasma concentrations of both MDMA and bupropion and enhanced mood effects but also result in lower cardiac stimulation.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA; "ecstasy") is a popular recreational drug that acts by releasing dopamine (DA), norepinephrine (NE), and serotonin (5-HT) through their corresponding transporters (Verrico et al., 2007; Hysek et al., 2012d). The present study (ClinialTrials.gov #NCT01771874; http://www.clinicaltrials.gov/ct2/show/NCT01771874) was designed to contribute to elucidation of the mechanism of action of MDMA in humans. Specifically, we explored the modulatory role of DA in the psychotropic effects of MDMA by using bupropion pretreatment to block MDMA-induced DA release. Dopamine transporter inhibition prevents the release of DA through the DA transporter induced by MDMA or other amphetamines (Verrico et al., 2008; Simmler et al., 2013b). Dopamine mediates the reinforcing addictive effects of psychostimulants, but its role in the drug-induced subjective effects of different psychostimulants, such as euphoria, is less clear (Wise, 2008). Bupropion inhibits the DA transporter, less potently the NE transporter, but not the 5-HT transporter (Richelson and Pfenning, 1984; Andersen, 1989; Stahl et al., 2004). Using previously published methods (Simmler et al., 2013b), we also confirmed that bupropion inhibited the human DA, NE, and 5-HT transporter with IC_{50} values of 1.6, 18, and >100 μM, respectively. Bupropion has been shown to inhibit the amphetamine- and methamphetamine-induced release of DA in vitro (Gruner et al., 2009; Simmler et al., 2013b) and decrease methamphetamine self-administration in rats (Reichel et al., 2009) and monkeys (Schindler et al., 2011). Bupropion also reduced methamphetamine-induced subjective and cardiostimulant effects in humans (Newton et al., 2005, 2006) and may reduce drug use in subsets of methamphetamine users (Elkashef et al., 2008; Heinzerling et al., 2014). These findings suggest a role for DA in both the rewarding and subjective effects of methamphetamine. In contrast, the role of DA in the acute mechanism of action of MDMA is less clear. In preclinical studies, DA receptor gene deletion in mice had minimal effects on MDMA-induced behavioral changes (Risbrough et al., 2006), and DA transporter inhibition did not alter the acute response to MDMA in rhesus monkeys (Verrico et al., 2008). In contrast, 5-HT and NE have been well documented to mediate most of the acute psychotropic and physiologic effects of MDMA in humans (Liechti et al., 2000; Liechti and Vollenweider, 2000; Farre et al., 2007; Hysek et al., 2011, 2012d). In particular, inhibition of both the 5-HT and NE transporters with duloxetine, which prevents the MDMA-induced release of 5-HT and NE through their respective transporters, almost

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ABBREVIATIONS: AUC, area under the plasma concentration-time curve; AUEC, area under the effect-time curve; [^{11}C]β-CIT-FE, *N*-(2-fluoroethyl)-2β-carbomethoxy-3β-(4-iodophenyl)-nortropane; DA, dopamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; 5-HT, 5-hydroxytryptamine (serotonin); LLOQ, lower limit of quantification; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NE, norepinephrine; VAS, Visual Analog Scale.

completely abolished the subjective and cardiostimulant response to MDMA in humans (Hysek et al., 2012d).

We previously showed that the DA and NE transporter inhibitor methylphenidate did not alter the subjective response to MDMA in healthy subjects, which is consistent with DA having no relevant contribution to the psychotropic effects of MDMA in humans (Hysek et al., 2014). Because methylphenidate produced substantial subjective effects on its own (Hysek et al., 2014), however, this prior study was inconclusive. In contrast to methylphenidate, bupropion is a more potent DA transporter inhibitor than NE transporter inhibitor (Stahl et al., 2004) and is more selective for DA compared with methylphenidate, which blocks the DA and NE transporters with equal potency (Simmler et al., 2014). Additionally, bupropion has been proposed to bind to the substrate recognition site on the DA transporter similarly to MDMA, whereas psychoactive DA transporter ligands, such as methylphenidate and cocaine, may interact with a different binding site on the DA transporter (Heal et al., 2014). Bupropion reaches a high brain-to-plasma ratio and brain concentrations above its IC_{50} value for DA transporter inhibition (Stahl et al., 2004). Thus, we investigated the effects of pretreatment with bupropion or placebo on the pharmacodynamics and pharmacokinetics of MDMA in healthy subjects. We hypothesized that bupropion pretreatment would prevent the MDMA response to the extent that the effects of MDMA in humans depend on an interaction with the DA and NE transporters. Specifically, we expected bupropion to reduce the mood and cardiostimulant effects of MDMA through DA and NE transporter inhibition, respectively.

Bupropion inhibits CYP2D6 (Kotlyar et al., 2005), which inactivates MDMA to 4-hydroxy-3-methoxymethamphetamine (HMMA; de la Torre et al., 2012). Therefore, bupropion can be expected to increase plasma concentrations of MDMA. Furthermore, CYP2B6, which metabolizes bupropion to hydroxybupropion (Jefferson et al., 2005), is also involved in the minor metabolic pathway of MDMA to form the psychoactive metabolite 3,4-methylenedioxyamphetamine (MDA) by *N*-demethylation, in addition to the involvement of CYP1A2 and CYP3A4 (Kreth et al., 2000). Thus, the competitive inhibition of CYP2B6 by bupropion might alter the conversion of MDMA to MDA, and MDMA may inhibit the metabolism of bupropion. Thus, in addition to pharmacodynamic interactions at the DA and NE transporters, complex pharmacokinetic interactions between bupropion and MDMA are also likely and were examined in the present study.

Materials and Methods

Study Design

This study used a double-blind, placebo-controlled, crossover design with four experimental test sessions (placebo–placebo, bupropion–placebo, placebo–MDMA, and bupropion–MDMA) that were performed in a counterbalanced order according to a Latin-square randomization design. The washout periods between sessions were at least 10 days. The study was conducted at the University Hospital of Basel in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice and approved by the Ethics Committee of the Canton of Basel, Switzerland, and the Swiss Agency for Therapeutic Products (Swissmedic). The study was registered at ClinicalTrials.gov (NCT01771874). The predefined primary endpoint of the study was the effect of bupropion on "good drug effects" associated with MDMA. All subjects provided written informed consent and were paid for their participation.

Subjects

Sixteen healthy white subjects (eight men and eight women) with a mean ± S.D. age of 24.3 ± 2.2 years and a body mass index of 22.7 ± 2.1 kg/m^2 were recruited from the University of Basel campus. The inclusion criterion was 18–45 years of age. Subjects with a personal or first-degree-relative history of psychiatric disorders or chronic or acute physical illness were excluded as previously described (Hysek et al., 2012a). Additional exclusion criteria were tobacco smoking (>10 cigarettes/day) and a lifetime history of using illicit drugs more than five times, with the exception of past cannabis use. Six subjects had used MDMA once previously. Drug use histories are shown in Table 1. Subjects who used any illicit drugs, including cannabis, within the past 2 months or during the study period were excluded. We performed drug tests at screening and before each test session using TRIAGE 8 (Biosite, San Diego, CA). Female participants were investigated during the follicular phase of their menstrual cycle (days

TABLE 1
Prevalence of drug use
Values are times used in life except for tetrahydrocannabinol (THC), coffee, alcohol, and smoking.

Subject	Sex	Age	MDMA	Amphetamine	Cocaine	LSD	Psilocybin	THC	Coffee	Alcohol Use	Smoking	Smoking
		yr	*pills*					*joints/yr*	*cups/day*	*drinks/wk*	*cigarettes/day*	*yr*
1	M	25	0	0	0	0	0	Never	0.0	0	0	0
2	F	23	0	0	0	0	0	<1	4.5	1	0	0
3	M	25	0	0	0	0	0	<1	0.0	0	0	0
4	F	22	1	0	0	0	0	<1	1.0	2	0	0
5	F	27	0	0	0	0	0	<1	2.0	3	0	0
6	M	27	1	0	2	0	0	10–15	1.5	3	5	10
7	M	22	0	0	0	0	0	5–10	2.0	3	0	0
8	M	25	0	0	0	0	0	Never	2.0	2	0	0
9	F	27	1	0	0	0	0	<1	2.0	3	0	0
10	F	27	1	0	0	0	0	<1	3.5	3	3	10
11	M	25	1	0	0	0	0	<1	3.0	5	0	0
12	M	25	1	0	0	0	0	5–10	2.0	3	0	0
13	M	24	0	0	0	1	1	2–4	2.5	7	0	0
14	F	20	0	0	0	0	0	<1	1.0	5	0	0
15	F	22	0	0	0	0	0	2–4	0.0	2	0	0
16	F	22	0	0	0	0	0	<1	2.0	1	0	0

LSD, lysergic acid diethylamide.

2–14) to account for cyclic changes in the reactivity to amphetamines (White et al., 2002). All subjects were genotyped (Hicks et al., 2013) and phenotyped (Trojan et al., 2012) for CYP2D6 activity. The study included 13 extensive, three intermediate, and no poor CYP2D6 metabolizers (genotyping and phenotyping congruent).

Study Outline

The study included a prescreening telephone interview, a screening visit, four whole-day test sessions with a next-day follow-up, and an end-of-study visit. Bupropion or placebo was administered daily for 7 days before each of the test sessions. The test sessions began at 7:45 AM. An indwelling intravenous catheter was placed in an antecubital vein for blood sampling, and the subjects completed baseline measurements of mood and vital signs. Bupropion (300 mg p.o.) or placebo was administered at 8:00 AM. MDMA (125 mg p.o.) or placebo was administered at 10:00 AM. A standardized lunch was served at 12:30 PM, and the subjects were sent home at 6:00 PM. On the day after each test session, the participants returned to the research ward at 10:00 AM for assessment of subjective and adverse effects and collection of the 24-hour blood sample.

Drugs

±MDMA hydrochloride ($C_{11}H_{15}NO_2$, Lipomed AG, Arlesheim, Switzerland) was prepared as gelatin capsules (100 and 25 mg). Identical-looking placebo (mannitol) capsules were prepared. MDMA was administered in a single absolute dose of 125 mg, corresponding to 1.8 ± 0.2 mg/kg body weight (mean ± S.D.). Bupropion tablets [150 mg, Wellbutrin XR 150 mg (GlaxoSmithKline, Munchenbuchsee, Switzerland) and mannitol as filler] were encapsulated within opaque gelatin capsules, and identical placebo (mannitol pill with mannitol filler) capsules were prepared. Bupropion was administered once daily at a dose of 150 mg for 3 days, followed by administration of 300 mg of bupropion once daily for 4 days before the test days. A similar regimen is used to initiate smoking cessation treatment with bupropion. The subjects were reminded by a phone text message to ingest the capsules in the morning, and medication containers were checked to confirm that the first seven doses of bupropion were administered. The last dose of bupropion (300 mg) was administered onsite under supervision 2 hours before MDMA was administered. Similar pretreatment regimens with bupropion produced 26% DA transporter occupancy as measured by [^{11}C]β-CIT-FE [*N*-(2-fluoroethyl)-2β-carbomethoxy-3β-(4-iodophenyl)-nortropane] positron emission tomography 3 hours after the last dose of bupropion (Learned-Coughlin et al., 2003) and reduced the subjective response to methamphetamine in humans (Newton et al., 2006).

Outcome Measures

Vital Signs. Blood pressure, heart rate, and core body temperature were assessed repeatedly 2 hours and 1 hour before and 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 hours after MDMA or placebo administration as previously described (Hysek and Liechti, 2012). The cardiovascular measures were performed in duplicate after a resting time of at least 10 minutes. The averages were calculated for the analyses.

Pupillometry. Pupillometry was performed 2 hours and 1 hour before and 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 hours after drug administration. Pupil function was measured using a PRL-200 infrared pupillometer (NeurOptics, Irvine, CA) under dark-light conditions of 6.1 ± 1 lux as described previously (Hysek and Liechti, 2012). The dark-adapted pupil diameter was measured in both eyes, and the average values were used for analyses.

Endocrine Measures. Plasma levels of prolactin and cortisol were measured at baseline and 2 hours after MDMA or placebo administration using radioimmunoassays (Hysek et al., 2012b). Plasma levels of oxytocin were measured before and 1 hour and 2 hours after administration of MDMA or placebo by radioimmunoassay (Neumann et al., 2013). Concentrations of circulating catecholamines, including epinephrine and NE, were measured at baseline and 1 hour and 2 hours after administration of MDMA or placebo using ultra-performance liquid chromatography–tandem mass spectrometry (Dunand et al., 2013). Plasma epinephrine levels are mainly derived from the adrenal medulla, whereas the entrance of NE into the plasma represents an overflow by sympathetic nerves (Esler et al., 1990; Eisenhofer et al., 1995). Circulating NE is therefore considered an indicator of sympathetic system activation. DA in plasma does not derive from DA but mostly from NE neurons (Goldstein and Holmes, 2008). Nevertheless, we measured DA levels in plasma because there are no data on the effects of MDMA on DA plasma levels.

Adverse Effects. Adverse effects were assessed using the 66-item list of complaints (Zerssen, 1976) before and 5 and 24 hours after MDMA or placebo administration. The scale yields a total adverse effects score, reliably measuring physical and general discomfort.

Psychometric Scales. Subjective effects were repeatedly assessed using previously described psychometric scales. Visual Analog Scales (VASs; Hysek et al., 2011) were administered 2 hours and 1 hour before and 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 24 hours after administration of MDMA or placebo.

Pharmacokinetics. Blood samples for the determination of MDMA, MDA, HMMA, bupropion, hydroxybupropion, and hydrobupropion were collected 2 hours before and 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 24 hours after MDMA or placebo administration. Plasma MDMA, MDA, and HMMA concentrations were determined using high-performance liquid chromatography–tandem mass spectrometry as described previously (Hysek et al., 2012a, 2013). Bupropion, hydroxybupropion, and hydrobupropion were included into the analytical method, and slight modifications were made. In brief, the chromolith speed ROD RP-18e (50 × 4.6 mm; Merck, Darmstadt, Germany) analytical column was replaced by a Luna PFP (2) column (50 × 2 mm; Phenomenex, Torrance, CA), and bupropion, hydroxybupropion, and hydrobupropion were added as additional analytes. Peak symmetry was improved by online dilution of the samples with water supplemented with 0.1% formic acid. Threohydrobupropion and erythrohydrobupropion were quantified together as hydrobupropion because the isomeric metabolites coeluted in chromatography and were indistinguishable in mass spectrometry. The performance of the method was monitored using quality-control samples at the lower limit of quantification (LLOQ) and at two to four other concentrations that covered the entire calibration range. The LLOQ values were 1 ng/ml for MDMA, MDA, HMMA, and hydroxybupropion, 5 ng/ml for bupropion, and 0.1 ng/ml for hydrobupropion. The interassay precision was <15% (LLOQ: 20%), and the interassay accuracy ranged from 85% to 115% (LLOQ: 80%–120%) for all the analytes.

Statistical and Pharmacokinetic Analyses

Peak effects (E_{max}) and peak changes from baseline (ΔE_{max}) were determined for repeated measures. E_{max} and ΔE_{max} values were analyzed by two-way repeated-measures analysis of variance, with MDMA (MDMA versus placebo) and bupropion (bupropion versus placebo) as within-subjects factors, using Statistica 12 software (StatSoft, Tulsa, OK). Tukey's post hoc comparisons were performed based on significant main effects or interactions. The criterion for significance was $P < 0.05$. Pharmacokinetic data were analyzed using noncompartmental models. Peak plasma concentration (C_{max}) and the time to reach maximal plasma concentration (T_{max}) were obtained directly from the observed concentration-time curves. For MDMA, HMMA, and bupropion, the terminal elimination rate constant (λ_z) was estimated by log-linear regression after semilogarithmic transformation of the data using at least three data points of the terminal linear phase of the concentration-time curve. The terminal elimination half-life ($t_{1/2}$) was calculated using λ_z and the equation $t_{1/2} = \ln 2/\lambda_z$. Determining the $t_{1/2}$ values for MDA, hydroxybupropion, and hydrobupropion was not possible because of their long $t_{1/2}$, which would require a longer sampling time. The area under the plasma concentration-time

TABLE 2

Pharmacodynamic effects

Values are mean ± S.E.M. of peak changes from baseline (ΔE_{max}) or peak effects (E_{max}) in 16 subjects.

		Placebo–Placebo	Bupropion–Placebo	Placebo–MDMA	Bupropion–MDMA	Main Effect of MDMA		Main Effect of Bupropion		Bupropion × MDMA Interaction	
						$F_{1,15}$	P value	$F_{1,15}$	P value	$F_{1,15}$	P value
Vital signs											
SBP (mm Hg)	ΔE_{max}	9.3 ± 1.5	9.4 ± 1.5	33.1 ± 2.5***	27.7 ± 1.5***	91.22	<0.001	2.76	NS	2.62	NS
DBP (mm Hg)	ΔE_{max}	5.3 ± 0.9	6.1 ± 1.3	17.7 ± 1.3***	16.9 ± 1.4***	72.17	<0.001	0.00	NS	0.50	NS
Heart rate (beats/min)	ΔE_{max}	5.6 ± 1.1	6.6 ± 1.5	30.2 ± 3.1***	18.5 ± 2.2***,###	57.97	<0.001	12.38	0.003	13.97	0.002
Body temperature (°C)	ΔE_{max}	0.39 ± 0.07	0.31 ± 0.05	0.63 ± 0.09	0.66 ± 0.07*	18.26	<0.001	0.19	NS	0.71	NS
Pupillometry											
Pupil size (mm)	E_{max}	6.90 ± 0.14	6.90 ± 0.17	7.74 ± 0.13***	7.76 ± 0.14***	103.06	<0.001	0.04	NS	0.06	NS
Hormones											
Prolactin (mU/l)	ΔE_{max}	−219 ± 34	−211 ± 50	441 ± 191**	388 ± 196**	16.45	0.001	0.11	NS	0.09	NS
Cortisol (nmol/l)	ΔE_{max}	−414 ± 37	−425 ± 81	150 ± 51***	106 ± 48***	63.08	<0.001	0.20	NS	0.26	NS
Oxytocin (pg/ml)	ΔE_{max}	−0.9 ± 0.6	−1.0 ± 0.4	86 ± 15***	79 ± 17***	44.56	<0.001	0.11	NS	0.10	NS
Epinephrine (nmol/l)	ΔE_{max}	0.04 ± 0.02	0.03 ± 0.03	0.5 ± 0.1***	0.4 ± 0.07***	48.68	<0.001	0.72	NS	0.20	NS
Norepinephrine (nmol/l)	ΔE_{max}	−0.32 ± 0.13	0.10 ± 0.12	0.96 ± 0.19***	0.33 ± 0.12*,#	20.94	<0.001	1.89	NS	9.57	0.007
Dopamine (nmol/l)	ΔE_{max}	0.03 ± 0.02	−0.01 ± 0.02	0.08 ± 0.03	0.06 ± 0.03	2.61	NS	1.60	NS	0.84	NS
List of complaints (total score)											
Acute adverse effects	≤5 h	2.2 ± 0.7	2.6 ± 1.0	15.9 ± 1.8***	15.1 ± 1.0***	97.57	<0.001	0.07	NS	0.50	NS
Subacute adverse effects	≤24 h	1.3 ± 0.6	2.4 ± 0.8	8.4 ± 1.5***	7.9 ± 1.2***	33.38	<0.001	0.14	NS	1.12	NS
Subjective effects											
Visual Analog Scale (%max)											
Any drug effect	ΔE_{max}	0.3 ± 0.3	1.4 ± 1.0	75 ± 5***	84 ± 3***	508.82	<0.001	2.81	NS	2.35	NS
	$\Delta AUEC_{0-8h}$	0.2 ± 0.2	3.6 ± 3.3	191 ± 22***	261 ± 26***,#	118.97	<0.001	7.34	0.02	6.56	0.02
Good drug effect	ΔE_{max}	3.1 ± 3.1	0.9 ± 0.9	69 ± 5***	77 ± 5***	236.03	<0.001	0.93	NS	2.62	NS
	$\Delta AUEC_{0-8h}$	1.6 ± 1.6	0.5 ± 0.5	167 ± 23***	244 ± 35***,#	62.26	<0.001	6.71	0.02	6.69	0.02
Drug high	ΔE_{max}	0.0 ± 0.0	0.2 ± 0.2	63 ± 7***	76 ± 5***	167.20	<0.001	3.67	NS	3.66	NS
	$\Delta AUEC_{0-8h}$	0.0 ± 0.0	0.1 ± 0.1	131 ± 26***	188 ± 37***,#	29.43	<0.001	5.22	0.04	5.22	0.04
Drug liking	ΔE_{max}	2.4 ± 2.4	3.3 ± 3.3	72 ± 6***	76 ± 6***	242.54	<0.001	0.85	NS	0.38	NS
	$\Delta AUEC_{0-8h}$	1.2 ± 1.2	2.5 ± 2.5	173 ± 22***	245 ± 39***,#	61.50	<0.001	4.57	0.049	4.61	0.049
Stimulated	ΔE_{max}	0.0 ± 0.0	0.4 ± 0.4	61 ± 8***	68 ± 7***	102.27	<0.001	0.64	NS	0.56	NS
	$\Delta AUEC_{0-8h}$	0.0 ± 0.0	0.4 ± 0.4	144 ± 27***	193 ± 40***	28.69	<0.001	3.74	NS	3.69	NS

AUE, area under the effect-time curve; DBP, diastolic blood pressure; NS, not significant; SBP, systolic blood pressure.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with placebo-placebo; #$P < 0.05$; ###$P < 0.001$ compared with placebo-MDMA.

curve (AUC) and area under the effect-time curve (AUEC) were calculated using the linear trapezoidal rule.

Results

Autonomic Effects. Peak effects and statistics are summarized in Table 2. MDMA increased blood pressure, heart rate, and body temperature (Fig. 1, A–D). Bupropion significantly reduced the MDMA-induced increase in heart rate (Fig. 1C), but it did not significantly affect the increases in blood pressure (Fig. 1, A and B) or body temperature (Fig. 1D) induced by MDMA. Bupropion did not alter the mydriatic effect of MDMA on pupillary function (Table 2).

Endocrine Effects. MDMA increased plasma concentrations of prolactin, cortisol, oxytocin, epinephrine, and NE compared with placebo. Bupropion significantly reduced the MDMA-induced increases in the plasma concentrations of NE but not of other hormones (Table 2). Plasma levels of DA were very low and in 75% of the measurements were below the lower limit of detection (< 0.1 nM). None of the treatments altered DA plasma concentrations.

Adverse Effects. The acute (up to 5 hours) and subacute (up to 24 hours) adverse effects of MDMA were not altered by bupropion (Table 2). Frequently reported acute adverse effects of placebo–MDMA and bupropion–MDMA were lack of appetite (n = 13 for both), perspiration (n = 11 and 12, respectively), tremor (n = 8 and 11, respectively), restlessness (n = 10 and 7, respectively), dry mouth (n = 14 and 12, respectively), and bruxism (n = 13 for both). Subacute adverse effects included headache (n = 12 and 8, respectively), tiredness (n = 9 and 10, respectively), lack of appetite (n = 8 and 9, respectively), difficulty concentrating (n = 7 and 6, respectively), dry mouth (n = 5 and 9, respectively), and bruxism (n = 6 and 10, respectively). No severe adverse effects were reported.

Subjective Effects. Peak effects and statistics are summarized in Table 2. MDMA increased VAS ratings for "any drug effect," "good drug effect," "drug high," "drug liking," and "stimulated" (Fig. 2, A–E). Bupropion enhanced the positive mood effects of MDMA, reflected by a significant increase in AUEC values and a nonsignificant increase in maximal effect ratings and in the bupropion–MDMA condition compared with the placebo–MDMA condition for VAS scales ratings for "any drug effect," "good drug effect," "drug high," and "drug liking" (Fig. 2, A–D; Table 2). MDMA-induced increases in "stimulation" were not significantly altered by bupropion (Fig. 2E).

Pharmacokinetics. The drug and metabolite concentration-time curves are shown in Fig. 3. The pharmacokinetic parameters are shown in Table 3. Bupropion pretreatment significantly increased the plasma concentration of MDMA ($C_{max,}$ $P < 0.01$; $AUC_{0–8}$, $P < 0.001$; $AUC_{0–24,}$ $P < 0.001$) and prolonged its $t_{1/2}$ ($P < 0.01$). In contrast, bupropion pretreatment significantly decreased the plasma concentrations of MDA (C_{max}, $P < 0.01$; $AUC_{0–8,}$ $P < 0.001$) and HMMA ($C_{max,}$ $P < 0.001$; $AUC_{0–8,}$ $P < 0.001$; $AUC_{0–24,}$ $P < 0.001$) and prolonged the $t_{1/2}$ and T_{max} of HMMA (both $P < 0.001$). MDMA significantly increased the plasma concentration of bupropion ($C_{max,}$ $P < 0.05$; $AUC_{0–8,}$ $P < 0.001$; $AUC_{0–24,}$ $P < 0.01$). MDMA

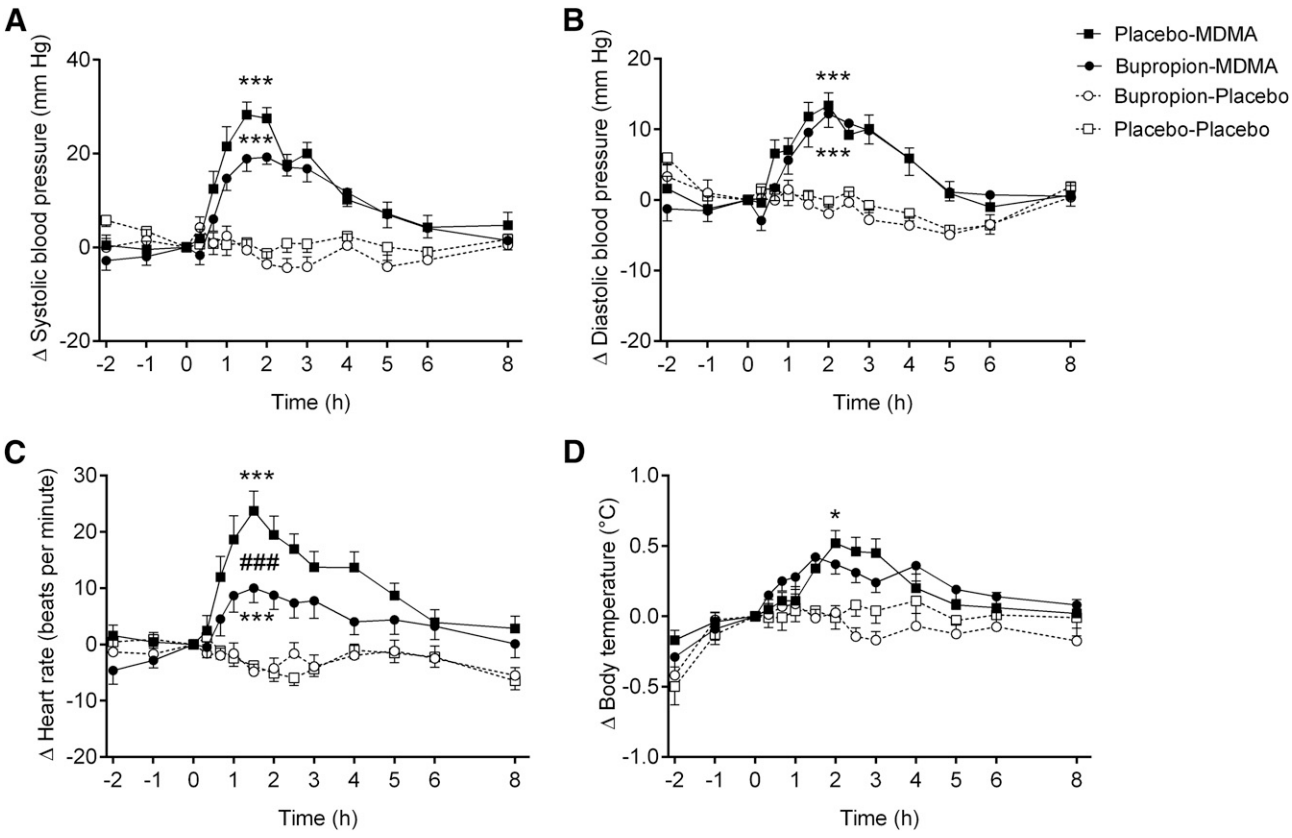

Fig. 1. Bupropion reduced the MDMA-induced an increase in heart rate (C) compared with placebo–MDMA but not in the blood pressure (A and B) or body temperature (D) response to MDMA. MDMA or placebo was administered at t = 0 hour. Data are expressed as mean ± S.E.M. in 16 subjects. $^{*}P < 0.05$; $^{***}P < 0.001$ for significant differences in the maximal effects compared with placebo-placebo; $^{###}P < 0.001$ compared with placebo–MDMA.

also slightly increased the C_{max} of hydrobupropion ($P < 0.05$), but it had no effect on the concentration of hydroxybupropion.

The MDMA concentration-effect plot (Fig. 4.) shows that higher subjective effects were reached early during the drug response in the bupropion–MDMA condition compared with the placebo–MDMA condition at similar MDMA concentrations consistent with a dynamic drug interaction. Thus, bupropion did not reduce the MDMA response taking into account any pharmacokinetic interactions.

Discussion

In the present study, bupropion reduced the heart rate response to MDMA and prolonged its subjective effects. We hypothesized that bupropion prevents the pharmacodynamic effects of MDMA to the extent that these effects depend on DA and NE release. Bupropion reduced the MDMA-induced increases in circulating NE, which is a marker of sympathetic system activation, and the cardiostimulant effects of MDMA similarly to the selective NE transporter inhibitor reboxetine (Hysek et al., 2011). The blockade of α- and β-adrenergic receptors by carvedilol reduced the heart rate and blood pressure response to MDMA (Hysek et al., 2012c). Together, these findings indicate that NE mediates the cardiostimulant effects of MDMA. In contrast, blocking the DA transporter with bupropion did not reduce and actually prolonged the positive mood effects of MDMA. Thus, DA does not appear to be a critical mediator of the subjective effects of MDMA. Otherwise, a reduction in the mood response would have been expected. Methylphenidate, which inhibits the DA transporter more potently than bupropion (Simmler et al., 2013b; Heal et al., 2014), did not attenuate the subjective effects of MDMA (Hysek et al., 2014). In contrast, several studies showed that the subjective effects of MDMA in humans are significantly reduced by 5-HT (Liechti et al., 2000; Farre et al., 2007; Tancer and Johanson, 2007) and NE (Hysek et al., 2011) transporter inhibition and almost completely blocked by dual 5-HT and NE transporter inhibition (Hysek et al., 2012d). Additionally, bupropion did not alter adverse effects of MDMA, in contrast to 5-HT (Liechti and Vollenweider, 2000) or 5-HT and NE transporter inhibitors (Hysek et al., 2012d). These clinical mechanistic studies support the view that 5-HT and NE are the primary mediators of the acute psychological effects of MDMA, whereas DA appears to be less relevant. 5-HT receptor agonists fully substituted for the discriminative stimulus effects of MDMA in rats, but methamphetamine did not (Mori et al., 2014). Unlike MDMA, methamphetamine predominantly acts on the DA system (Simmler et al., 2013a,b), and bupropion reduced the subjective effects of methamphetamine (Newton et al., 2006), consistent with a more important role for DA in the action of methamphetamine.

How bupropion prolonged the subjective response to MDMA in the present study is unclear. Bupropion has previously been shown to similarly enhance the positive subjective effects of cocaine (Oliveto et al., 2001). Bupropion increased the plasma concentration of MDMA, and this pharmacokinetic bupropion–MDMA interaction could partially explain the enhanced psychotropic effects of MDMA induced by bupropion. However, the concentration-effect relationship indicated that bupropion also increased the subjective effects of MDMA irrespective of its increasing effect on MDMA plasma concentrations.

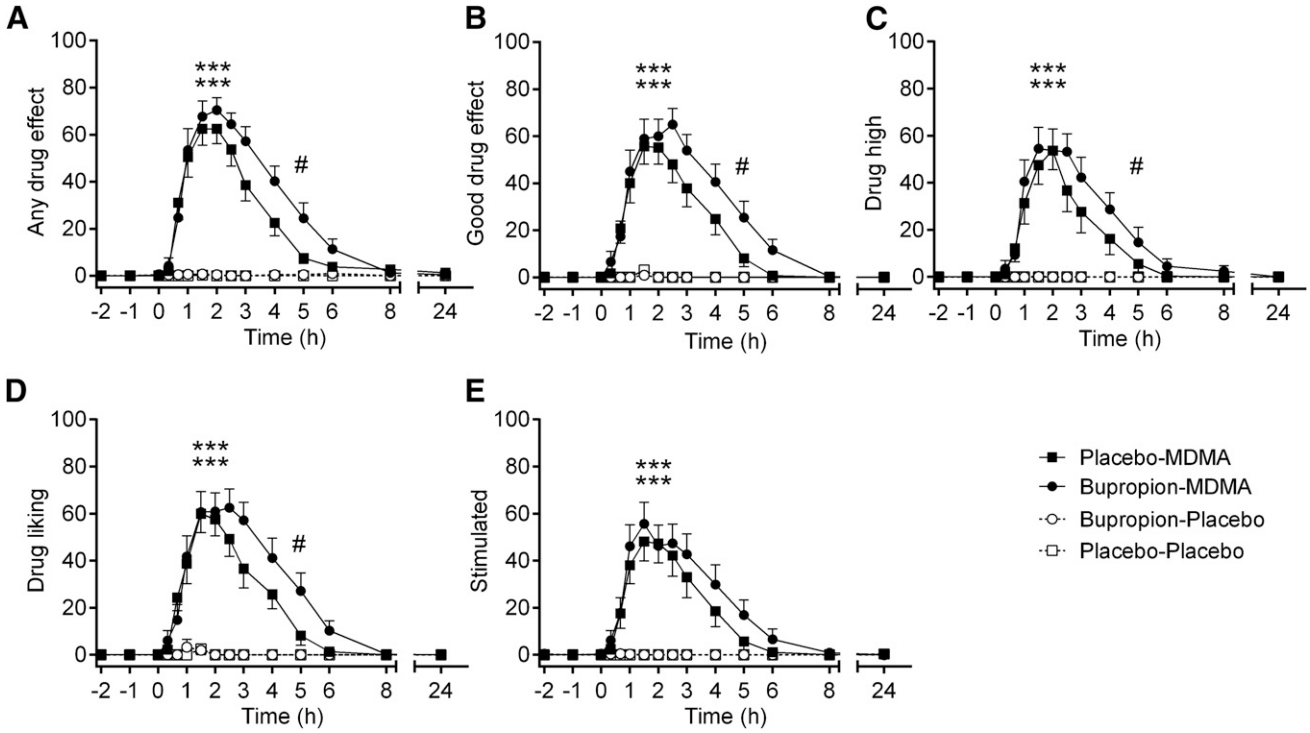

Fig. 2. Bupropion pretreatment enhanced the subjective mood effects of MDMA. The MDMA-induced area under the effect-concentration curves for VAS scale ratings for (A) "any drug effect," (B) "good drug effect," (C) "drug high," and (D) "drug liking," but not (E) "stimulation," were all significantly greater after bupropion–MDMA compared with MDMA alone ($^{\#}P < 0.05$ compared with placebo–MDMA). MDMA or placebo was administered at $t = 0$ hour. Values are expressed as mean ± S.E.M. in 16 subjects. $^{***}P < 0.001$ compared with placebo–placebo.

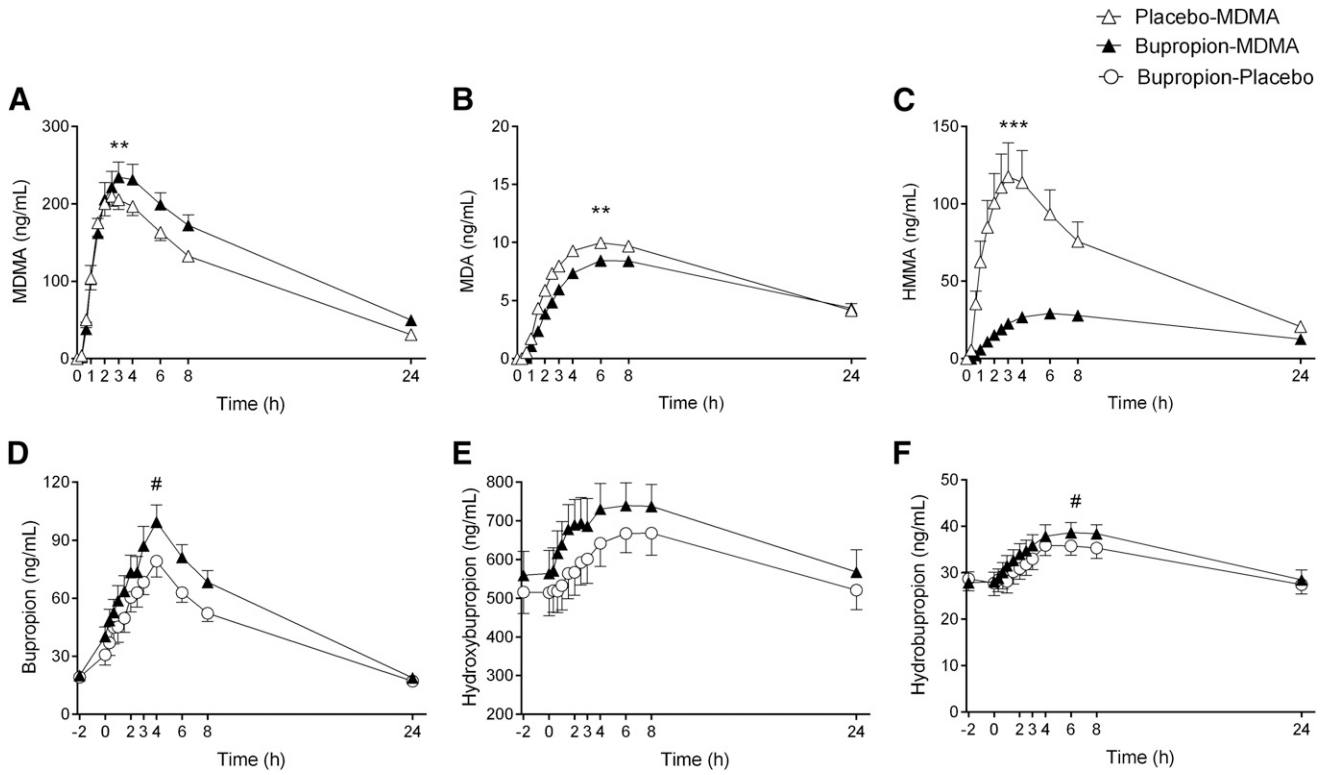

Fig. 3. Plasma concentration-time profiles. (A) Bupropion significantly increased the plasma concentration of MDMA (C_{max}, AUC_{0-8}, and AUC_{0-24}) and (B) significantly decreased the plasma concentrations of the MDMA metabolites MDA (C_{max} and AUC_{0-8}) and HMMA (C) (C_{max}, AUC_{0-8}, and AUC_{0-24}). (D) MDMA significantly increased the plasma concentrations of bupropion (C_{max}, AUC_{0-8}, and AUC_{0-24}) and hydrobupropion (F) (C_{max}) but had no significant effect on hydroxybupropion concentration (E). The pharmacokinetic parameters are shown in Table 2. MDMA or placebo was administered at $t = 0$ hour, and the last pretreatment administration of bupropion occurred at $t = -2$ hours. Values are expressed as mean ± S.E.M. in 16 subjects. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences between placebo–MDMA and bupropion–MDMA. $^{\#}P < 0.05$ indicates significant difference between bupropion-placebo and bupropion–MDMA.

Bupropion increased the C_{max} of MDMA by 15%, increased the AUC_{0-24h} of MDMA by 30%, and decreased the C_{max} and AUC_{0-24h} of the MDMA metabolite HMMA by 75% and 66%, respectively. Because MDMA is primarily metabolized to HMMA by CYP2D6 (Segura et al., 2005; de la Torre et al., 2012), the effects of bupropion on the pharmacokinetics of MDMA and HMMA are explained by CYP2D6 inhibition. Bupropion, and particularly erythro-hydrobuprion and threo-hydrobupropion have previously been shown to inhibit CYP2D6 (Jefferson et al., 2005; Kotlyar et al., 2005; Reese

TABLE 3

Pharmacokinetic parameters of MDMA and bupropion and metabolites

Values are mean ± S.E.M. in 16 healthy subjects.

	C_{max} (ng/ml)	AUC_{0-8}	AUC_{0-24}	$t_{1/2}$	T_{max}
		ng/ml·h			*h*
MDMA					
Placebo–MDMA	231 ± 14	1262 ± 72	2576 ± 156	7.4 ± 0.4	2.5 ± 0.2
Bupropion–MDMA	264 ± 13**	1535 ± 67***	3428 ± 144***	9.2 ± 0.7**	3.1 ± 0.2
MDA					
Placebo–MDMA	10.3 ± 0.5	59.2 ± 3.1	170 ± 11		6.1 ± 0.3
Bupropion–MDMA	8.8 ± 0.5**	46.8 ± 2.7***	149 ± 8.5		6.8 ± 0.3
HMMA					
Placebo–MDMA	123 ± 22	711.2 ± 99	1482 ± 247	8.5 ± 0.5	3.5 ± 0.3
Bupropion–MDMA	29.6 ± 3.3***	169 ± 18***	492 ± 54***	15.1 ± 1.1***	6.0 ± 0.3***
Bupropion					
Bupropion–placebo	93.2 ± 7.5	486 ± 36	1030 ± 69	10.3 ± 0.9	5.1 ± 0.5
Bupropion–MDMA	110 ± 8.3$^{\#}$	615 ± 40$^{\#\#\#}$	1313 ± 84$^{\#\#}$	8.7 ± 0.8	5.8 ± 0.4
Hydroxybupropion					
Hydroxybupropion–placebo	748 ± 63	4976 ± 337	14490 ± 1273		7.3 ± 0.6
Hydroxybupropion–MDMA	793 ± 62	5613 ± 486	16056 ± 1336		7.1 ± 0.6
Hydrobupropion					
Hydrobupropion–placebo	37.4 ± 2.1	267 ± 18	769 ± 51		7.0 ± 0.6
Hydrobupropion–MDMA	40.9 ± 2.0$^{\#}$	287 ± 17	822 ± 47		7.7 ± 0.5

$^{**}P < 0.01$; $^{***}P < 0.001$ compared with placebo–MDMA; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with bupropion–placebo.

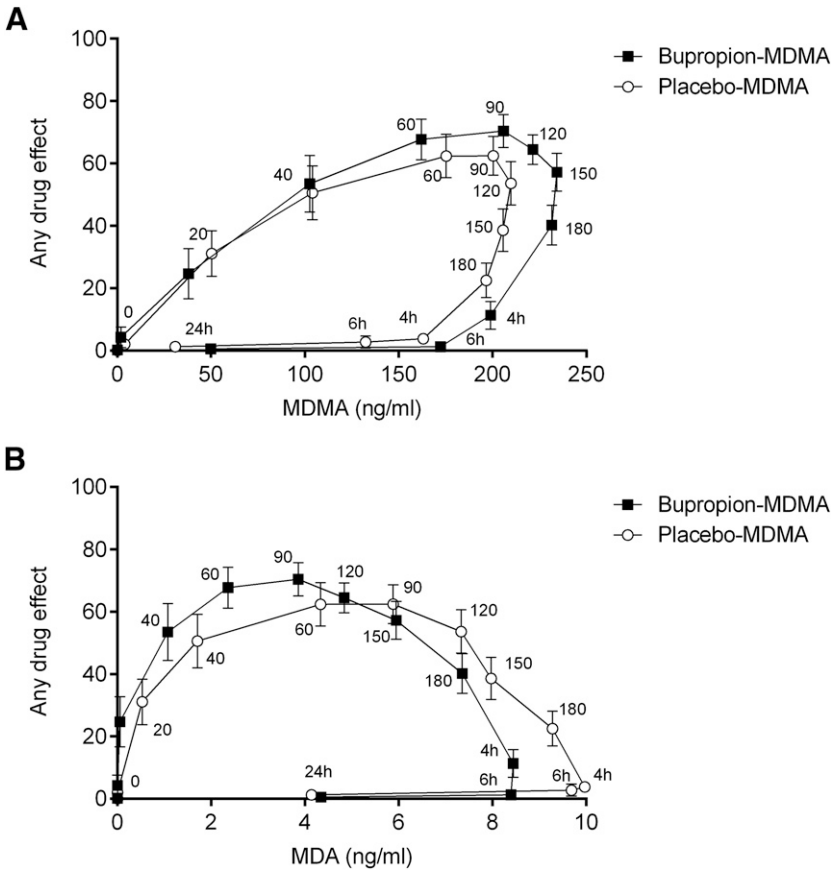

Fig. 4. Subjective effects of (A) MDMA and (B) MDA plotted against plasma concentrations of MDMA. The values are expressed as the means and S.E.M. values in 16 subjects. The time of sampling is noted next to each point in minutes or hours after MDMA administration. (A) Up to the peak response to MDMA, slightly higher subjective drug effects were reported after bupropion–MDMA compared with placebo–MDMA at a given MDMA concentration. Contrary to our hypothesis bupropion did not reduce the subjective response to MDMA. Note the rapid acute tolerance to the subjective effects of MDMA.

et al., 2008). Other CYP2D6 inhibitors, including paroxetine, reboxetine, and duloxetine (Farre et al., 2007; Hysek et al., 2011, 2012d), also increased the plasma levels of MDMA and lowered HMMA concentrations (Farre et al., 2007; Hysek et al., 2012d) to an extent similar to that of bupropion in the present study. Interestingly, bupropion also decreased plasma levels of MDA in the present study. Pure CYP2D6 inhibition would shift MDMA metabolism from HMMA formation to MDA formation, resulting in higher plasma MDA levels as previously reported after reboxetine or duloxetine pretreatment (Hysek et al., 2011, 2012d). Thus, in the present study, the minor metabolic pathway of MDMA to MDA by CYP2B6, CYP3A4, and CYP1A2 (Kreth et al., 2000) was also inhibited, possibly via competitive CYP2B6 inhibition by bupropion (Hesse et al., 2000).

MDMA also altered the pharmacokinetics of bupropion. Specifically, MDMA increased the C_{max} of bupropion by 18% and AUC_{0-24} by 27%, together with slight increases in hydrobupropion and hydroxybupropion. Hydrobupropion is formed by nonmicrosomal carbonyl reductase, and hydroxybupropion is formed by CYP2B6 (Hesse et al., 2000; Jefferson et al., 2005). How MDMA increased the plasma levels of bupropion and its metabolites is unclear. CYP2B6 inhibition by MDMA could explain the increase in plasma concentration of bupropion, but a decrease in hydroxybupropion would be expected. The effects of MDMA on the pharmacokinetics of bupropion could be clinically relevant because MDMA enhanced the exposure to bupropion and its metabolites, and all the metabolites of bupropion are also pharmacologically active (Damaj et al., 2004; Jefferson et al., 2005; Zhu et al., 2012).

The present study had a few limitations. First, only one dose regimen for bupropion and one single, relatively high dose of MDMA were used. Second, bupropion treatment produced DA transporter occupancy in the human striatum of only 26% (Learned-Coughlin et al., 2003). This occupancy may not have been sufficient to prevent MDMA from interacting with the DA transporter. It was important, however, to use a DA transporter inhibitor with no psychoactive effects, as shown for bupropion in the present study and previously (Peck and Hamilton, 1983; Oliveto et al., 2001) and in contrast to methylphenidate (Hysek et al., 2014; Schmid et al., 2014). Third, both MDMA and bupropion exhibit stereoselective metabolism (Kharasch et al., 2008; Steuer et al., 2014). The analytical method used in the present study was not stereoselective. The analytical methods are currently being developed to further address interactions between MDMA and bupropion enantiomers.

In conclusion, bupropion–MDMA coadministration resulted in prolonged positive mood effects but lower cardiostimulant effects than MDMA alone. Bupropion increased the plasma concentration of MDMA and vice versa. These findings indicate that NE contributes to the cardiovascular effects of MDMA, with no evidence that DA mediates the subjective effects of MDMA.

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Authorship Contributions

Participated in research design: Schmid, Rickli, Hysek, Liechti.

Conducted experiments: Schmid, Rickli, Schaffner, Duthaler, Grouzmann, Hysek.

Performed data analysis: Schmid, Rickli, Liechti.

Wrote or contributed to the writing of the manuscript: Schmid, Liechti.

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Paper 2

Monoamine transporter and receptor interaction profiles of a new series of designer cathinones.

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Monoamine transporter and receptor interaction profiles of a new series of designer cathinones

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ABSTRACT

Psychoactive β-keto amphetamines (cathinones) are sold as "bath salts" or "legal highs" and recreationally abused. We characterized the pharmacology of a new series of cathinones, including methedrone, 4-methylethcathinone (4-MEC), 3-fluoromethcathinone (3-FMC), pentylone, ethcathinone, buphedrone, pentedrone, and N,N-dimethylcathinone. We investigated norepinephrine (NE), dopamine (DA), and serotonin (5-HT) uptake inhibition using human embryonic kidney 293 (HEK 293) cells that express the respective human monoamine transporter, the drug-induced efflux of NE, DA, and 5-HT from monoamine-preloaded cells, and binding affinity to monoamine transporters and receptors. All of the cathinones were potent NE uptake inhibitors but differed in their DA *vs.* 5-HT transporter inhibition profiles and monoamine release effects. Methedrone was a more potent 5-HT than DA transporter inhibitor and released NE and 5-HT similar to para-methoxymethamphetamine (PMMA), para-methoxyamphetamine (PMA), 4-methylthioamphetamine (4-MTA), and 3,4-methylenedioxymethamphetamine (MDMA). 4-MEC and pentylone equipotently inhibited all of the monoamine transporters and released 5-HT. Ethcathinone and 3-FMC inhibited NE and DA uptake and released NE, and 3-FMC also released DA similar to N-ethylamphetamine and methamphetamine. Pentedrone and N,N-dimethylcathinone were non-releasing NE and DA uptake inhibitors as previously shown for pyrovalerone cathinones. Buphedrone preferentially inhibited NE and DA uptake and also released NE. None of the cathinones bound to rodent trace amine-associated receptor 1, in contrast to the non-β-keto-amphetamines. None of the cathinones exhibited relevant binding to other monoamine receptors. In summary, we found considerable differences in the monoamine transporter interaction profiles among different cathinones and compared with related amphetamines.

1. Introduction

The illicit stimulant market has become complex. From 2005 to 2011, 34 novel cathinone-type designer drugs have been detected in the European Union (EMCDDA, 2013). These drugs are typically available online as "legal highs," "bath salts," or "research chemicals" (EMCDDA, 2013) and have been added to users' club drug repertoires (Moore et al., 2013), resulting in unknown health risks. Intoxication with different cathinone derivatives has been reported worldwide (Borek and Holstege, 2012; James et al., 2011; Prosser and Nelson, 2012; Zuba et al., 2013). Structurally, the novel designer cathinones are all substituted amphetamines, but their pharmacology and toxicology show considerable variability (Dal Cason et al., 1997; Simmler et al., 2013) and are not known in many cases. Recent studies characterized the *in vitro* pharmacological profiles of cathinones, including ethylone, mephedrone, naphyrone, butylone, methylone, flephedrone, cathinone, methcathinone, pyrovalerone, and 3,4-methylenedioxypyrovalerone (MDPV; Baumann et al., 2013; Eshleman et al., 2013; Iversen et al., 2013; Lopez-Arnau et al., 2012; Simmler et al., 2013). These preclinical studies allow comparisons of the pharmacological mechanisms of action of novel designer drugs with well-known amphetamines, including methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA).

Abbreviations: DA, dopamine; DAT, dopamine transporter; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine (serotonin); MDMA, 3,4-methylenedioxymethamphetamine; MDPV, 3,4-methylenedioxypyrovalerone; 4-MTA, 4-methylthioamphetamine; NE, norepinephrine; NET, norepinephrine transporter; PMA, para-methoxyamphetamine; PMMA, para-methoxymethamphetamine; SERT, serotonin transporter; TAAR, trace amine-associated receptor.

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Several novel cathinones with unknown pharmacological characteristics have emerged. Methedrone is the β-keto-substituted analog of para-methoxymethamphetamine (PMMA). PMMA and para-methoxyamphetamine (PMA) are para-ring-substituted amphetamine derivatives sold as Ecstasy, alone or in combination with MDMA (Brunt et al., 2012). PMA and PMMA epidemics have been described worldwide for many years (Johansen et al., 2003; Lurie et al., 2012; Vevelstad et al., 2012). PMA and PMMA use has been associated with high morbidity and mortality particularly attributable to hyperthermia (Brunt et al., 2012; Lurie et al., 2012; Refstad, 2003). Methedrone is found in bath salt products (Marinetti and Antonides, 2013), and it may be associated with a high risk for mortality (Wikstrom et al., 2010), similar to PMA. PMA inhibits the serotonin (5-hydroxytrypatmine [5-HT]) uptake transporter (SERT) and induces 5-HT release (Callaghan et al., 2005) like MDMA. In animals, PMMA and PMA produce effects similar to MDMA, but they are more potent and lack amphetamine-like stimulant effects in rodent drug discrimination studies (Dukat et al., 2002; Glennon et al., 2007). Pharmacological data on methedrone are unavailable. 4-Methylthioamphetamine (4-MTA) is the methylthio analog of PMA and also a SERT inhibitor (Huang et al., 1992) and 5-HT releaser (Gobbi et al., 2008; Huang et al., 1992). 4-MTA produces MDMA-like effects in animals and humans (Winstock et al., 2002) and is typically used by Ecstasy users (Winstock et al., 2002). Fatalities possibly linked to 5-HT syndrome have been described (De Letter et al., 2001).

4-Methylethcathinone (4-MEC) is reported to be available over the Internet as "NRG-2" (Brandt et al., 2010) and is a substitute for mephedrone (Zuba and Byrska, 2013). 4-MEC inhibits the dopamine (DA) and norepinephrine (NE) transporters (DAT and NET) and SERT (Iversen et al., 2013). Whether 4-MEC is also a monoamine releaser is currently unknown.

3-Fluoromethcathinone (3-FMC) has been detected in legal highs (Archer, 2009). 3-FMC was shown to have pronounced locomotor stimulant and ataxic effects in mice (Marusich et al., 2012), but its pharmacological profile is currently unknown. The pharmacology of its structural isomer 4-fluoromethcathinone (flephedrone) has recently been described (Eshleman et al., 2013; Simmler et al., 2013).

Ethcathinone was detected in a patient who presented with severe hyponatremia and seizures (Boulanger-Gobeil et al., 2012). Ethcathinone is the β-keto analog of *N*-ethylamphetamine, which is similar to methamphetamine but contains an *N*-ethyl-group. Ethcathinone is a rat NET, DAT, and SERT inhibitor and also releases NE and 5-HT but not DA from rat synaptosomes (Yu et al., 2000). *N*-Ethylamphetamine releases NE and also released 5-HT and DA with lower potency from rat synaptosomes (Tessel and Rutledge, 1976). We found no data on the effects of ethcathinone on human monoamine transporters.

Pentedrone, buphedrone, and pentylone are recently identified novel designer cathinones (Maheux and Copeland, 2012; Westphal et al., 2012; Zuba and Byrska, 2013). Buphedrone was detected in "Vanilla Sky" and other "legal high" pills as a frequent substitute for mephedrone in eastern Europe (Zuba et al., 2013). Intoxication with buphedrone and its recreational use have recently been described (Zuba et al., 2013). Pentylone has been detected in "legal high" samples from the Internet (Brandt et al., 2011) and head shops (Westphal et al., 2012), and fatalities associated with "bath salts" have been reported in the United States (Marinetti and Antonides, 2013). Buphedrone and pentedrone are the α-ethyl and α-pentyl β-keto analogs of methamphetamine, respectively. Pentedrone is structurally identical to pentylone but without the MDMA-like 3,4-methylenedioxy group (Fig. 1). Pentylone is a β-keto-analog of MDMA, similar to methylone and butylone (Simmler et al., 2013), and contains an α-pentyl group. *N,N*-dimethylcathinone is the *N*-methylated β-keto analog of methamphetamine, and it has similar but less potent stimulant effects (Dal Cason et al., 1997). We found no data on the molecular pharmacology of pentylone, pentedrone, buphedrone, or *N,N*-dimethylcathinone.

In the present study, we characterized the *in vitro* pharmacology of a novel series of cathinones, including methedrone, 4-MEC, 3-FMC, pentylone, ethcathinone, buphedrone, pentedrone, and *N,N*-dimethylcathinone, and the profiles of the non-β-keto amphetamine analog comparator drugs 4-MTA, PMA, PMMA, MDMA, *N*-ethylamphetamine, and methamphetamine, complementing our previous characterization of this class of designer drugs (Simmler et al., 2013). As amphetamine derivatives, these drugs were expected to interact predominantly with monoamine transporters and receptors. We determined the potencies of the drugs to inhibit the human NET, DAT, and SERT. We tested whether the drugs induce the transporter-mediated release of NE, DA, and 5-HT and characterized the binding affinities of the drugs for monoamine transporters, α_1 and α_2 adrenergic receptors, dopamine D_1–D_3 receptors, 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, the histamine H_1 receptor, and the trace amine-associated receptor 1 ($TAAR_1$).

2. Methods

2.1. Drugs

The drugs were supplied by Lipomed (Arlesheim, Switzerland) or Cayman chemicals (Ann Arbor, MI, USA) as hydrochloride salts (purity > 98.5%). Racemic drugs were used, with the exception of D-methamphetamine. All of the radioligands were obtained from Perkin Elmer (Schwerzenbach, Switzerland) or Anawa (Wangen, Switzerland), with the exception of [^{3}H]RO5166017, which was synthesized at Roche (Basel, Switzerland).

2.2. Monoamine uptake transporter inhibition

The 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 in detail (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 concentrations) to initiate the uptake transport of the labeled monoamines at room temperature. Uptake was stopped after 10 min, and the cells were separated from the buffer by brief centrifugation through silicone oil (Hysek et al., 2012). Centrifugation tubes were frozen in liquid nitrogen and cut to separate the cell pellet from the silicon oil and the assay buffer layers. The cell pellet was 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 <10% of total uptake. The data were fit by nonlinear regression to variable-slope sigmoidal dose–response curves, and IC_{50} values were calculated using Prism (GraphPad, San Diego, CA). DAT/SERT ratios were calculated as 1/DAT IC_{50}: 1/SERT IC_{50}.

2.3. Transporter-mediated monoamine release

We studied 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 HEK-SERT cells with 10 nM [^{3}H]5-HT, HEK-DAT cells with 10 nM [^{3}H]DA and 1 μM unlabeled DA, and HEK-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 drugs at concentrations of 100 μM, with the exception of 4-MTA at the NET for which 10 μM was used. We incubated the HEK-SERT and HEK-DAT cells for 15 min and the HEK-NET cells for 45 min at 37 °C by 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 and methamphetamine. 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 substrate that diffuses out of the cells and reuptake inhibition (Rosenauer et al., 2013; Scholze et al., 2000), was assessed using the transporter inhibitors nisoxetine (HEK-NET cells), citalopram (HEK-SERT cells), and mazindol (HEK-DAT cells) at 10 μM. Thus, these uptake inhibitors served as negative control conditions. Methamphetamine and MDMA were used as comparator compounds that are known to induce monoamine release in this

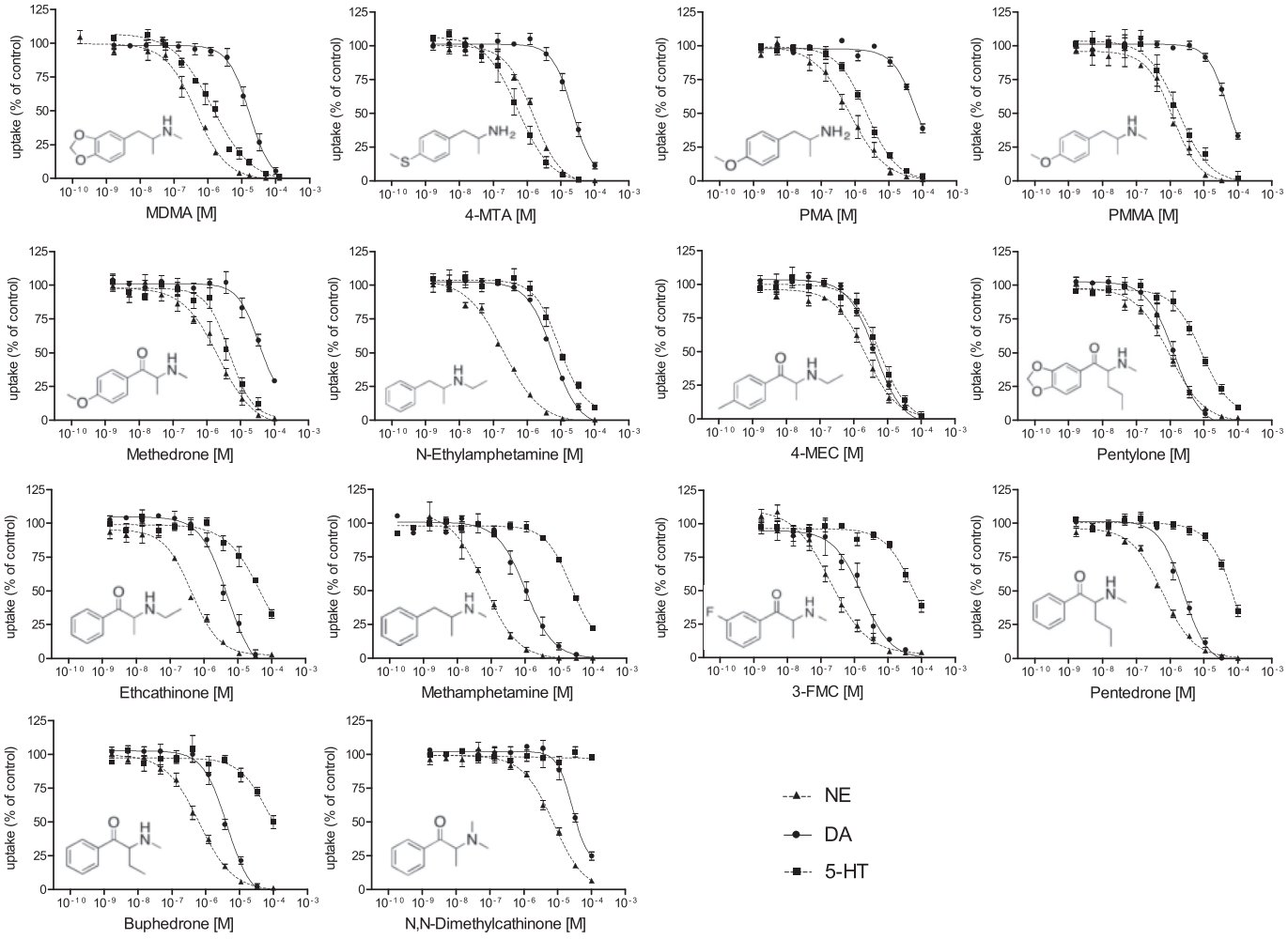

Fig. 1. Monoamine uptake inhibition presented as dose–response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as the mean ± SEM of 3–5 independent experiments. The lines show the data fit by nonlinear regression. IC_{50} values are shown in Table 2. The radioactivity in the cells (100%) amounted to 3718 ± 240 dpm for NE, 7200 ± 993 dpm for DA, and 8191 ± 1068 dpm for 5-HT (mean ± SEM, $n = 42$).

assay (positive control in each experiment; Simmler et al., 2013). All of the conditions were normalized to radioactive counts of the assay buffer control condition. We then used analysis of variance followed by Dunnett's test to compare the effects of the drug with the negative control condition (i.e., the activity of the transport inhibitors nisoxetine, citalopram, and mazindol is considered nonspecific release). Drugs that induced significantly higher maximal monoamine efflux compared with the respective transporter inhibitors, which induced slight nonspecific release, were considered monoamine releasers. The assays allow qualitative classification of a drug as a releaser or non-releaser but not quantitative comparisons between transporters.

Table 1
Monoamine transporter inhibition.

	NET	DAT	SERT	DAT/SERT ratio
	IC50 [μM] (95% CI)	IC50 [μM] (95% CI)	IC50 [μM] (95% CI)	Ratio (95% CI)
4-MTA[a]	1.52 (1.3–1.9)	22 (15–32)	0.54 (0.37–0.80)	0.02 (0.01–0.05)
PMA[a]	0.80 (0.50–1.0)	71 (60–83)	2.37 (2.0–2.9)	0.03 (0.02–0.05)
PMMA[a]	1.20 (0.75–1.8)	49 (18–135)	1.77 (1.1–2.9)	0.04 (0.01–0.16)
MDMA[a,b]	0.45 (0.33–0.60)	17 (12–24)	1.36 (1.0–2.0)	0.08 (0.04–0.16)
Methedrone	2.24 (1.4–3.5)	35 (15–79)	4.73 (3.2–6.9)	0.14 (0.04–0.46)
N-Ethylamphetamine[a]	0.20 (0.15–0.27)	5.86 (4.8–7.1)	8.77 (6–13)	1.5 (0.9–2.7)
4-MEC	2.23 (1.6–3.2)	4.28 (3.4–5.4)	7.93 (3.5–18)	1.85 (0.6–5.3)
Pentylone	0.99 (0.72–1.4)	1.34 (1.0–1.7)	8.37 (5.4–13)	6.2 (3.2–13)
Ethcathinone	0.44 (0.34–0.56)	5.00 (3.7–6.8)	48 (4–529)	9.6 (0.6–142)
Methamphetamine[a,b]	0.064 (0.04–0.09)	1.05 (0.74–1.5)	23 (14–40)	>10
3-FMC	0.19 (0.13–0.29)	1.7 (1.0–3.0)	56 (7–472)	>10
Buphedrone	0.65 (0.51–0.81)	4.24 (3.3–5.5)	70 (2–2700)	>10
Pentedrone	0.61 (0.52–0.72)	2.50 (2.0–3.2)	135 (5–3700)	>10
N,N-Dimethylcathinone	7.71 (5–12)	27 (21–36)	>500	>10

Values are means of three to five independent experiments and 95% confidence intervals (CI).
Drugs are ranked according to the DAT/SERT ratio = 1/DAT IC_{50}: 1/SERT IC_{50}.
[a] Non-beta-keto-amphetamine comparator compounds.
[b] Values from Simmler et al. (2013).

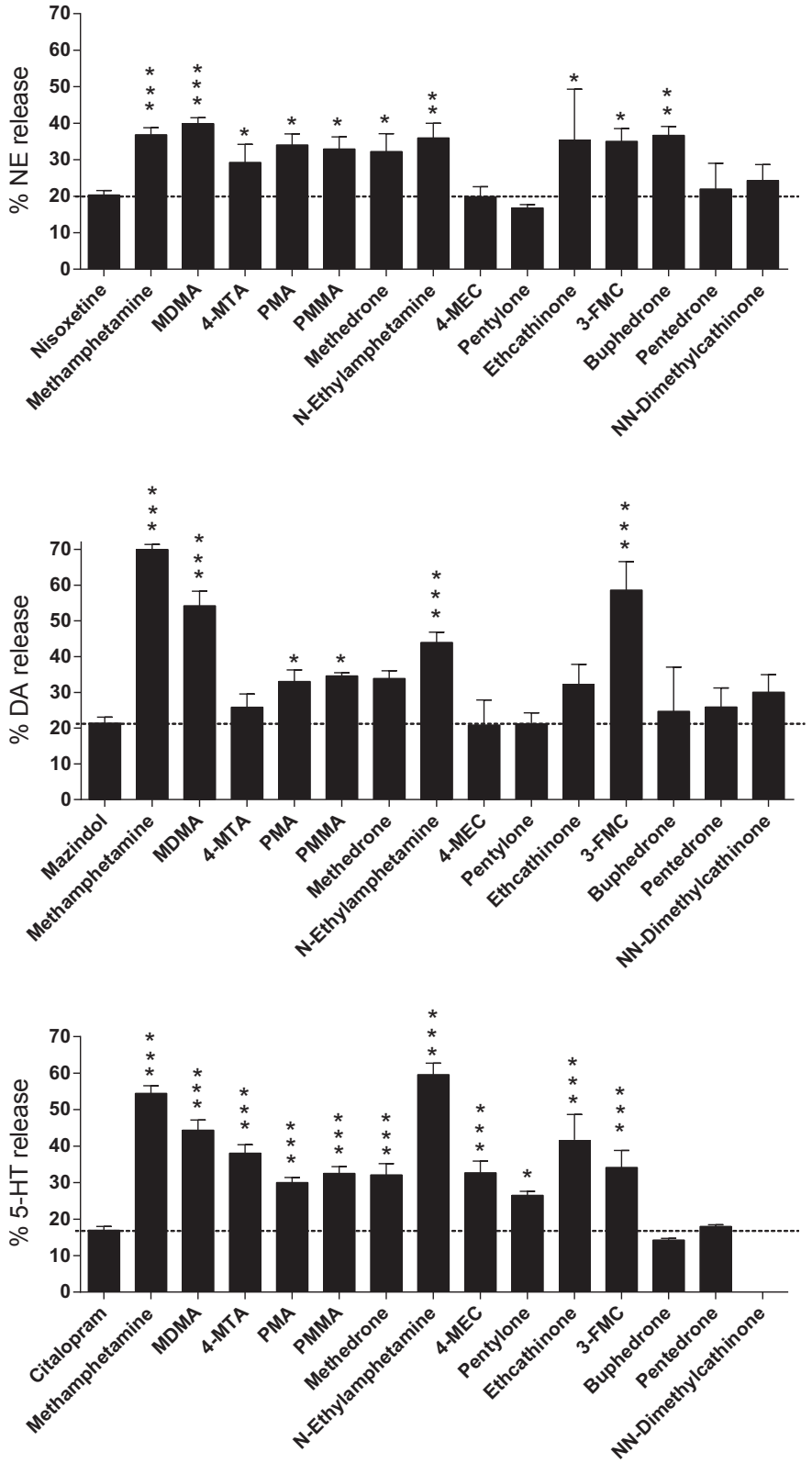

Fig. 2. Qualitative assessment of monoamine release. HEK 293 cells that expressed NET, DAT, and SERT were loaded with [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT, respectively, washed, and incubated with drugs. Monoamine release is expressed as the percent drug-induced reduction of monoamine cell content compared with DMSO vehicle (0% release, 100% activity in the cells). The radioactivity in the cells (0% release) amounted to 736 ± 24 dpm for NE, 2191 ± 66 dpm for DA, and 2872 ± 98 dpm for 5-HT (mean ± SEM, $n = 18$). 100% release would indicate that all monoamine was released from the cells (0% remaining activity in the cells). Non-releasing monoamine transporter blockers induce nonspecific "pseudo-efflux" (dashed line), which arises from substrate that diffuses out of the cells and reuptake inhibition. High concentrations of the test drugs were used (100 μM, except for 4-MTA at the NET [10 μM]). The assays allow the qualitative classification of a drug as a releaser or non-releaser but not quantitative comparisons between transporters. The data are expressed as the mean ± SEM of 3–4 independent experiments (3–7 experiments for MDMA and methamphetamine) with negative controls added in each experiment. Statistically, total release of the test drugs was compared with the nonspecific effects of the negative control drugs (nisoxetine for NE, mazindol for DA, and citalopram for 5-HT) using analysis of variance followed by Dunnett's test. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, significant effects compared with controls.

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 except for $TAAR_1$ receptors that were rat/mouse; 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 (8-OH-DPAT) 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 (DA D_1 receptor), [^{3}H]spiperone and spiperone (DA 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.

3. Results

3.1. Monoamine uptake transporter inhibition

The effects of the cathinones and comparator drugs on monoamine transporter function are presented in Fig. 1. The corresponding IC_{50} values for monoamine transport inhibition and DAT/SERT inhibition ratios are shown in Table 1. With the exception of *N,N*-dimethylcathinone, which was a weak NET and DAT inhibitor, all of the compounds shared potent effects as NET inhibitors, whereas their DAT and SERT inhibition potencies varied considerably, reflected by the wide range of DAT/SERT inhibition ratios. The para-ring-substituted compounds, including 4-MTA, PMA, PMMA, and methedrone, were potent SERT and NET inhibitors but weak DAT inhibitors, similar to MDMA. The DAT/SERT ratio was <0.5 for all of these MDMA-like drugs. Additionally, methedrone was the only cathinone in the present series of drugs with an MDMA-like profile and DAT/SERT ratio <1. Generally, with the exception of methedrone, all of the cathinones examined in this series preferentially inhibited the NET and DAT more than the SERT. 3-FMC, pentedrone, buphedrone, and *N,N*-dimethylcathinone exhibited very low potency at the SERT, and the DAT/SERT inhibition ratios were all >10. Ethcathinone was 10-fold less potent at the SERT *vs.* the DAT, whereas its non-β-keto analog ethylamphetamine was equipotent at the DAT and SERT, confirming that the addition of a β-keto group enhanced the DAT inhibitory properties over SERT inhibitory properties (Simmler et al., 2013). In contrast, ring substitutions enhanced serotonergic properties. 4-MEC, the 4-ring-methylated analog of ethcathinone, was 6-fold more potent at the SERT than ethcathinone. Similarly, the 3,4-ring methoxylated analog of pentedrone, pentylone, was at least 10-fold more potent at the SERT than pentedrone.

3.2. Transporter-mediated monoamine release

The drug effects on the transporter-mediated release of NE, DA, and 5-HT from transmitter-preloaded cells are depicted in Fig. 2. By means of this quantitative assessment at high drug concentrations, we identified whether the drugs induce specific NE, DA, and 5-HT release compared with the non-releasing controls nisoxetine, mazindol, and citalopram. All of the non-β-keto amphetamines and most of the cathinones tested in this series induced the release of at least one monoamine. Pentedrone and *N,N*-dimethylcathinone did not induce monoamine release and thus were pure uptake inhibitors. Methedrone and 3-FMC released all three monoamines similarly to all of the non-β-keto amphetamines methamphetamine, MDMA, PMMA, PMA, and *N*-ethylamphetamine. In contrast, ethcathinone and 4-MTA released NE and 5-HT but had no effect on DA release. 4-MEC and pentylone weakly released 5-HT but not DA or NE, whereas buphedrone released NE but not DA or 5-HT.

3.3. Binding affinities

Table 2 shows the binding profiles of the test drugs expressed as the potencies of the drugs (K_i) to inhibit radioligand binding to the NET, DAT, and SERT and different monoamine receptors. Importantly, none of the drugs exhibited very high affinity (<100 nM) for any of the monoamine transporters or human receptors. Submicromolar affinity (<1 μM) DAT interactions were found with the cathinones 4-MEC, pentylone, ethcathinone, and pentedrone but not non-β-keto amphetamines. Conversely, the non-β-keto amphetamines but not the cathinones showed affinity for the rat and mouse $TAAR_1$. Some of the test drugs showed relevant binding (<10 μM) to 5-HT_{1A} receptors (ethcathinone, *N*-ethylamphetamine, and methamphetamine), 5-HT_{2A} receptors (4-MEC, *N,N*-dimethylcathinone, 4-MTA, and MDMA), 5-HT_{2C} receptors (4-MEC, 3-FMC, *N,N*-dimethylcathinone, 4-MTA, and *N*-ethylamphetamine), and α_2 receptors (4-MTA, *N*-ethylamphetamine, and methamphetamine). None of the drugs tested bound to DA D_1–D_3 or histamine H_1 receptors.

The pure uptake inhibitors and releasers typically differed with regard to their K_i:IC_{50} ratio. Most of the releasers showed higher functional potency compared with binding potency, resulting in ratios >6 for the NET and >1 for the DAT and SERT, with some exceptions for drugs that released monoamines only at high drug concentrations (>10 μM).

4. Discussion

We characterized the monoamine receptor binding profiles and interactions with monoamine transporters of a series of seven novel designer cathinones that are components of “bath salts” or “legal highs.” The pharmacological profiles of the seven cathinone derivatives were compared with their non-β-keto analogs or related non-β-keto-substituted amphetamines. All of the cathinones inhibited monoamine transport as expected based on their amphetamine structure. However, considerable differences were found among these cathinones and compared with the non-β-keto amphetamines, similar to other cathinone derivatives (Baumann et al., 2013; Eshleman et al., 2013; Iversen et al., 2013; Rosenauer et al., 2013; Simmler et al., 2013).

First, the compounds markedly differed with regard to their serotoninergic *vs.* dopaminergic properties as expressed by DAT/SERT ratios. A low DAT/SERT ratio may be associated with a lower abuse potential of a drug (Baumann et al., 2011; Rothman and Baumann, 2006; Simmler et al., 2013; Wee et al., 2005), although lipophilicity, blood–brain barrier transport (Simmler et al., 2013), and pharmacokinetic factors are also involved. Second, some cathinones were pure uptake inhibitors, whereas others were substrate releasers. Third, the cathinones typically did not bind to rodent $TAAR_1$, in contrast to the non-β-keto-amphetamines.

4.1. Methedrone is a serotonergic cathinone comparable to para-(4)-substituted amphetamines and MDMA

Methedrone was the cathinone with the highest selectivity for the SERT in both the present and our previously studied series of compounds (Simmler et al., 2013) and also induced monoamine efflux, exhibiting a profile identical to MDMA. Methedrone,

therefore, is the most MDMA-like cathinone in terms of the monoamine transporter interaction profile. Both methedrone and PMMA are para-methoxy- or methylthio-amphetamines, similar to PMA and 4-MTA. PMA, PMMA, and 4-MTA have long been associated with particularly high clinical toxicity and many fatalities mostly attributable to 5-HT syndrome, hyperthermia, and associated multi-organ failure (De Letter et al., 2001; Johansen et al., 2003; Lurie et al., 2012; Vevelstad et al., 2012). Similarly, fatal methedrone intoxication was recently described (Wikstrom et al., 2010). The present study showed that all of these para-substituted amphetamines are potent NET and SERT inhibitors with low potency at the DAT, as previously shown for 4-MTA (Huang et al., 1992) and 4-trifluoromethylmethcathinone (Cozzi et al., 2013). These drugs released NE and 5-HT and at high concentrations (>10 μM) also released DA, as previously shown for 4-MTA, PMA, and 4-trifluoromethylmethcathinone *in vitro* and *in vivo* (Cozzi et al., 2013; Gobbi et al., 2008; Gough et al., 2002; Huang et al., 1992; Quinn et al., 2006; Sotomayor-Zarate et al., 2012). Importantly, the *in vivo* hyperthermic properties of the para-substituted amphetamines are stronger than those of MDMA (Daws et al., 2000) and have been associated with serotonergic and adrenergic receptor activation (Carmo et al., 2003). Therefore, hyperthermic complications should be of particular concern when these or similar para-substituted serotonergic cathinones are used recreationally.

4.2. 4-MEC and pentylone are equipotent DAT and SERT inhibitors and also release 5-HT

All of the cathinones, with the exception of methedrone, were more potent at the catecholamine transporters compared with the SERT. 4-MEC and pentylone inhibited all of the monoamine transporters with approximately equal potency that was similar to cocaine but also released 5-HT similar to MDMA. 4-MEC and pentylone are cathinones with profiles that are very similar to ethylone, butylone, and methylone (Simmler et al., 2013). 4-MEC, pentylone, and butylone (Simmler et al., 2013) released only 5-HT and not DA, differentiating them from the popular cathinone derivative mephedrone, which releases both 5-HT and DA *in vitro* (Hadlock et al., 2011; Simmler et al., 2013) and *in vivo* (Baumann et al., 2012; Kehr et al., 2011). The release of 5-HT by 4-MEC and pentylone may reduce the stimulant-like and addictive properties compared with mephedrone (Bauer et al., 2013). The monoamine uptake transporter inhibition profile for 4-MEC described in the present study is consistent with a previous report (Iversen et al., 2013), but no data are available for pentylone.

4.3. Ethcathinone and 3-FMC are methamphetamine-like cathinones

Ethcathinone was a weaker SERT inhibitor than its non-β-keto analog *N*-ethylamphetamine, confirming that cathinones are more potent inhibitors of the NET and DAT than the SERT compared with their non-β-keto amphetamine analogs (Iversen et al., 2013; Simmler et al., 2013). Ethcathinone and 3-FMC exhibited monoamine uptake transporter inhibition profiles that were similar to methamphetamine and flephedrone (Simmler et al., 2013). Additionally, ethcathinone released NE and 5-HT but not DA, as shown in previous studies using rat synaptosomes (Yu et al., 2000), and 3-FMC released all monoamines. Similar DA and 5-HT release has been documented for flephedrone, the positional isomer of 3-FMC (Simmler et al., 2013). Ethcathinone and 3-FMC can be classified as methamphetamine-like cathinones (Simmler et al., 2013), although ethcathinone did not release DA.

Table 2
Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$
4-MTA[a]	2.2 ± 1.3	6.7 ± 1.8	1.3 ± 0.2	>18	1.5 ± 0.4	1.8 ± 1.0	>6	7.4 ± 1.0	>12.5	>10	>16	>13	0.28 ± 0.04	0.04 ± 0.01
PMA[a]	28.2 ± 12.2	18.5 ± 3.9	7.1 ± 0.8	>20	11.2 ± 2.1	>13	>6	>25	>12.5	>10	>16	>13	0.66 ± 0.01	0.14 ± 0.1
PMMA[a]	14.4 ± 4.5	24.5 ± 3.9	10.1 ± 2.5	>20	13.6 ± 3.0	>13	>6	>25	>12.5	>10	14.0 ± 3.4	>13	1.25 ± 0.2	0.26 ± 0.1
MDMA[a,b]	26.8 ± 8.7	8.4 ± 3.3	13.0 ± 2.2	12.2 ± 0.8	5.9 ± 2.7	>13	>6	15.0 ± 10	>12.5	25.2 ± 12	>16	>13	0.37 ± 0.12	2.4 ± 1.1
Methedrone	25.3 ± 12.9	14.0 ± 7.8	17.0 ± 5.2	>20	>13	>13	>6	>25	>12.5	>10	>16	>13	>10	>10
N-Ethylamphetamine[a]	2.1 ± 0.8	1.1 ± 0.02	26.4 ± 6.1	3.4 ± 0.5	>13	6.0 ± 5.2	>6	2.9 ± 0.3	>12.5	>10	>16	>13	2.5 ± 1.4	>10
4-MEC	6.8 ± 0.5	0.89 ± 0.01	7.7 ± 0.4	>20	3.8 ± 1.6	5.2 ± 0.3	>6	>18	>12.5	>10	>16	>13	>10	>10
Pentylone	9.0 ± 2.4	0.24 ± 0.02	2.0 ± 0.5	>18	>13	>13	>6	>25	>12.5	>10	>16	>13	>10	>10
Ethcathinone	0.22 ± 0.1	0.88 ± 0.1	37.6 ± 9.0	8.5 ± 1.1	>13	9.3 ± 0.2	>6	15.5 ± 1.9	>12.5	>10	>16	>13	>10	>10
Methamphetamine[a,b]	3.0 ± 2.2	1.8 ± 0.7	24.6 ± 10.5	8.1 ± 0.8	>13	>13	>6	6.1 ± 1.6	>12.5	>10	>16	>13	0.35 ± 0.1	0.55 ± 0.2
3-FMC	5.0 ± 1.8	2.1 ± 0.4	36.1 ± 1.4	>18	>13	6.1 ± 2.2	>6	10 ± 2.2	>12.5	>10	>16	>13	>10	>10
Buphedrone	8.5 ± 4.2	1.3 ± 0.3	28.6 ± 18.7	>18	>13	>13	>6	23.9 ± 4.2	>12.5	>10	>16	>13	>10	>10
Pentedrone	4.5 ± 1.3	0.34 ± 0.03	17.3 ± 6.1	>18	>13	>13	>6	35.4 ± 16	>12.5	>10	>16	>13	>10	>10
N,N-Dimethylcathinone	23.8 ± 13	4.7 ± 1.1	>30	>18	6.5 ± 0.8	6.5 ± 0.8	>6	25.4 ± 11	>12.5	>10	>16	>13	>10	>10

NA, not assessed.
Values are K_i given as μM (mean ± SD, $n = 3–5$).
[a] Comparator compounds.
[b] Values for MDMA are from Simmler et al. (2013).

4.4. Pentedrone and N,N-dimethylcathinone are pure monoamine uptake inhibitors

Pentedrone and *N,N*-dimethylcathinone are uptake inhibitors only similar to cocaine and the pyrovalerone cathinones pyrovalerone, MDPV, and naphyrone (Baumann et al., 2013; Cameron et al., 2013; Eshleman et al., 2013; Simmler et al., 2013). These pure uptake inhibitors likely do not enter the intracellular space of the synapse via the transporter, which may be associated with less intracellular pharmacological effects and toxicity compared with substrate-type releasers (Eshleman et al., 2013). All non-releaser compounds, including pentedrone and *N,N*-dimethylcathinone, and the pyrovalerones pyrovalerone, MDPV, and naphyrone (Simmler et al., 2013) are tertiary amines, contain an α-propyl group, or share both structural characteristics, suggesting that these structures may prevent uptake by the transporters. Pentedrone and *N,N*-dimethylcathinone preferentially inhibited the catecholamine NET and DAT *vs*. SERT, although with lower potency and catecholamine transporter selectivity compared with pyrovalerone and MDPV (Simmler et al., 2013). 4- or 3,4-substitutions at the phenyl ring result in serotonergic selectivity, reflected by the low DAT/SERT inhibition ratio for MDMA, 4-MTA, PMA, PMMA, and methedrone. Similarly, the 4- or 3,4-phenyl ring-substituted compounds methylethcathinone, pentylone, and naphyrone but not MDPV (Simmler et al., 2013) have some activity at the SERT. None of the cathinone derivatives characterized in the present study exhibited very high potency at the DAT or the high DAT/SERT inhibition ratio >100 previously shown for MDPV (Baumann et al., 2013; Simmler et al., 2013) and associated with high reinforcing efficacy and compulsive use (Aarde et al., 2013; Watterson et al., in press). Nevertheless, the DAT/SERT inhibition ratios for buphedrone, pentedrone, and *N,N*-dimethylcathinone were all >10, similar to methamphetamine (Simmler et al., 2013), possibly indicating high abuse potential (Bauer et al., 2013).

Buphedrone is a catecholamine-selective transporter inhibitor, similar to pentedrone. Buphedrone did not release DA or 5-HT, similar to pentedrone, but it released NE. Buphedrone, therefore, has characteristics of both the pyrovalerone- and methamphetamine-like cathinones (Simmler et al., 2013).

4.5. Profiles of monoamine receptor and transporter binding

For the cathinones, submicromolar affinity interactions were observed with the monoamine transporters but not with other receptors, indicating that interactions with monoamine receptors likely do not contribute much to the *in vivo* effects of these drugs. We previously showed that cathinones exhibit approximately 10-fold lower affinity for $TAAR_1$ receptors compared with their non-β-keto analogs (Simmler et al., 2013). We extended this observation by showing that a series of additional cathinones consistently did not show relevant $TAAR_1$ binding affinity, in contrast to a series of additional non-β-keto amphetamines. Animal studies indicate that non-β-keto amphetamines, such as MDMA and methamphetamine, inhibit their own neurochemical and locomotor 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 in this new class of designer drugs compared with traditional amphetamines (Simmler et al., 2013).

Hallucinogens interact with 5-HT_1, 5-HT_{2A}, and 5-HT_{2C} receptors (Nichols, 2004), and several of the drugs tested in the present study showed low-affinity binding to these receptors. Consistent with our previous study (Simmler et al., 2013) and the work by others (Eshleman et al., 2013), no submicromolar binding was observed at 5-HT_{1A}, 5-HT_{2A}, or 5-HT_{2C} receptors for any of the drugs. Furthermore, others showed that several cathinones did not act as functional agonists or antagonists at these 5-HT receptors or that their functional potencies were very low (Eshleman et al., 2013). Actions at 5-HT receptors are therefore more likely to result from the drug-induced release of endogenous 5-HT rather than from direct interactions of the drugs with these 5-HT receptors as is the case with substituted phenethylamine hallucinogens (Nichols, 2004). In contrast, submicromolar affinity for 5-HT_{2B} receptors has been reported for naphyrone and mephedrone (Iversen et al., 2013). We did not assess 5-HT_{2B} receptor binding in the present study. 5-HT_{2B} receptors have been implicated in drug-associated cardiac valve fibrosis (Roth, 2007) and the behavioral effects of MDMA (Doly et al., 2008). Some previously described cathinones but none of the cathinones investigated in the present study exhibited low micromolar affinity for α_1 adrenergic receptors. Low-affinity α_2 receptor binding was observed for most non-β-keto amphetamines and some of the cathinones in the present study and our previous study (Simmler et al., 2013). Substrate releasers show higher transporter inhibition potency compared with binding affinity, resulting in K_i binding to IC_{50} uptake inhibition ratios >1 (Eshleman et al., 2013; Rudnick and Wall, 1992; Simmler et al., 2013). This phenomenon was also observed for most but not all monoamine releasers in the present study.

The present study has limitations. First, we used a static monoamine release assay (Rosenauer et al., 2013; Simmler et al., 2013; Verrico et al., 2007), which is confounded by back diffusion of the released monoamines. This assay is useful to determine whether a drug is a substrate releaser. Superfusion assays may be more suitable to also accurately determine the monoamine-release potency of the drugs (Eshleman et al., 2013). Second, stereoselective effects have been described for amphetamines, including substituted cathinones (Dal Cason et al., 1997), but we used only racemic drugs, similar to those recreationally used. Third, we did not investigate the effects of the drug on intracellular targets, such as the vesicular monoamine transporter or monoamine oxidase, which are affected by amphetamines (Eshleman et al., 2013; Green and El Hait, 1980) and contribute to their toxic effects (Steinkellner et al., 2011). Finally, we did not provide *in vivo* data. However, *in vitro* and *in vivo* pharmacological profiles were shown to be consistent for several cathinones (Baumann et al., 2012; Cozzi et al., 2013; Cozzi and Foley, 2003; Kehr et al., 2011).

Notably, no relevant interactions with the vesicular monoamine transporter were observed for a series of first-generation cathinones (Eshleman et al., 2013). In summary, the novel designer cathinones evaluated in the present study were all potent inhibitors of the NET, but marked differences were found in their DAT and SERT inhibition profiles and ability to also release monoamines.

Conflict of interest

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

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Paper 3

Pharmacological profiles of aminoindanes, piperazines, and pipradrol derivatives.

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Pharmacological profiles of aminoindanes, piperazines, and pipradrol derivatives

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ABSTRACT

Aminoindanes, piperazines, and pipradrol derivatives are novel psychoactive substances found in "Ecstasy" tablets as replacements for 3,4-methylenedioxymethamphetamine (MDMA) or substances sold as "ivory wave." The pharmacology of these MDMA- and methylphenidate-like substances is poorly known. We characterized the pharmacology of the aminoindanes 5,6-methylenedioxy-2-aminoindane (MDAI), 5-iodoaminoindane (5-IAI), and 2-aminoindane (2-AI), the piperazines meta-chlorophenylpiperazine (m-CPP), trifluoromethylphenylpiperazine (TFMPP), and 1-benzylpiperazine (BZP), and the pipradrol derivatives desoxypipradrol (2-diphenylmethylpiperidine [2-DPMP]), diphenylprolinol (diphenyl-2-pyrrolidinemethanol [D2PM]), and methylphenidate. We investigated norepinephrine (NE), dopamine (DA), and serotonin (5-hydroxytryptamine [5-HT]) uptake inhibition using human embryonic kidney 293 (HEK 293) cells that express the respective human monoamine transporters (NET, DAT, and SERT). We also evaluated the drug-induced efflux of NE, DA, and 5-HT from monoamine-preloaded cells and the binding affinity to monoamine transporters and receptors, including trace amine-associated receptor 1 ($TAAR_1$). 5-IAI and MDAI preferentially inhibited the SERT and NET and released 5-HT. 2-AI interacted with the NET. BZP blocked the NET and released DA. m-CPP and TFMPP interacted with the SERT and serotonergic receptors. The pipradrol derivatives were potent and selective catecholamine transporter blockers without substrate releasing properties. BZP, D2PM, and 2-DPMP lacked serotonergic activity and $TAAR_1$ binding, in contrast to the aminoindanes and phenylpiperazines. In summary, all of the substances were monoamine transporter inhibitors, but marked differences were found in their DAT *vs.* SERT inhibition profiles, release properties, and receptor interactions. The pharmacological profiles of D2PM and 2-DPMP likely predict a high abuse liability.

1. Introduction

New psychoactive substances [1] are constantly emerging on the illicit drug market. Many of these novel designer substances are amphetamine derivatives and typically marketed as "bath salts", "research chemicals" or "legal highs" via the Internet [2]. Pharmacological information is typically not available for these newly emerging designer substances. Interactions with the norepinephrine (NE), dopamine (DA), and serotonin (5-hydroxytryptamine [5-HT]) transporters (NET, DAT, and SERT, respectively) to block or release monoamines can be expected based on the amphetamine-like core structure of many of these substances. In addition, chemical modifications typically alter absolute or relative potencies at the NET and DAT relative to the SERT or substrate release properties, thereby affecting stimulant-like and reinforcing properties [3,4]. Additional interactions with the $5\text{-}HT_{2A}$ receptor may result in hallucinogenic-like actions. Substances that predominantly act on the NET and DAT have stimulant-like properties similar to amphetamine, whereas substances that mostly act on

Abbreviations: 2-AI, 2-aminoindane; BZP, 1-benzylpiperazine; DA, dopamine; DAT, dopamine transporter; D2PM, diphenyl-2-pyrrolidinemethanol; 2-DPMP, desoxypipradrol or 2-diphenylmethylpiperidine; HEK, human embryonic kidney; 5-IAI, 5-iodoaminoindane; m-CPP, meta-chlorophenylpiperazine; MDAI, 5,6-methylenedioxy-2-aminoindane; MDMA, 3,4-methylenedioxymethamphetamine; NE, norepinephrine; NET, norepinephrine transporter; 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; TAAR, trace amine-associated receptor; TFMPP, trifluoromethylphenylpiperazine..

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the SERT may have more "empathogenic" properties similar to 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) [4,5]. Assessing the *in vitro* pharmacological profiles of novel substances is a relatively rapid approach for gaining a first impression of their potential clinical effects and toxicology, in addition to user reports. Accordingly, the pharmacology of many novel designer cathinones ("bath salts" and "research chemicals") has recently been characterized *in vitro* [4,6–10]. The aim of the present study was to describe the effects on monoamine uptake and release of novel psychoactive substances that are not cathinones, but have been introduced into the illicit drug market as "legal highs" to typically mimic the subjective effects of MDMA or amphetamine-type stimulants. Aminoindanes, such as 5,6-methylenedioxy-2-aminoindane (MDAI) and 5-iodoaminoindane (5-IAI), became increasingly available over the Internet starting in 2010 as legal and, in the case of MDAI, allegedly less-neurotoxic alternatives to MDMA [11–13]. Piperazines have been used for more than a decade [14] and are commonly found in Ecstasy pills as substitutes for MDMA [15,16]. Toxicity associated with the use of "ivory wave," which contains the pipradrol derivatives desoxypipradrol (2-diphenylmethylpiperidine [2-DPMP]) or diphenylprolinol (diphenyl-2-pyrrolidinemethanol [D2PM]) was increasingly reported starting in 2010 [17–19]. The present study investigated the aminoindanes 2-aminoindane (2-AI), 5-IAI, and MDAI, the piperazines meta-chlorophenylpiperazine (m-CPP), trifluoromethylphenylpiperazine (TFMPP), and 1-benzylpiperazine (BZP), and the pipradrol derivatives D2PM and 2-DPMP (Fig. 1). Similar data on MDMA and other novel psychoactive substances have previously been published [4,6]. We determined the potencies of the compounds to inhibit the human NET, DAT, and SERT. We tested whether the compounds induce 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, 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, the histamine H_1 receptor, and trace amine-associated receptor 1 ($TAAR_1$). Most of the substances examined herein were previously studied using rodent transporters, but only a few were also studied using human transporters and receptors [7]. However, more comprehensive analyses are needed at both human transporters and receptors. Similar data on novel designer cathinones and classic stimulants, including amphetamine, methamphetamine, MDMA, and cocaine have previously been obtained using identical methods [4,6].

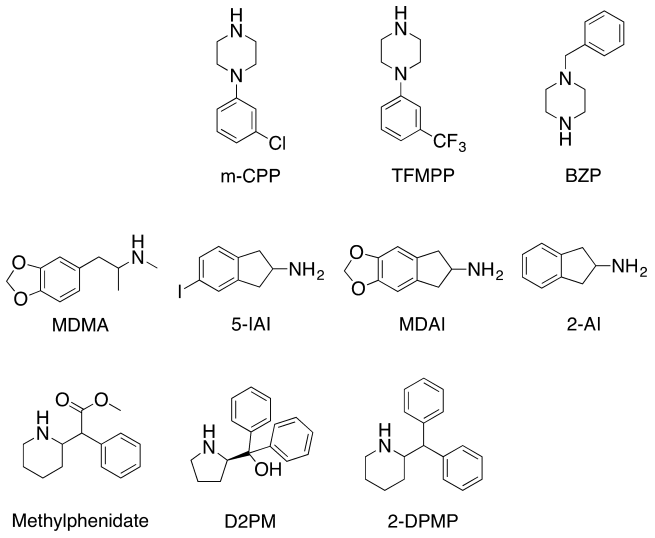

Fig. 1. Structures of novel psychoactive substances that mimic the effects of 3,4-methylenedioxymethamphetamine (MDMA) or methylphenidate. 2-Aminoindane (2-AI), 5-iodo-2-aminoindane (5-IAI), and 5,6-methylenedioxy-2-aminoindane (MDAI) are recreationally used aminoindanes. Meta-chlorophenylpiparazine (m-CPP), trifluoromethylphenylpiperazine (TFMPP), and 1-benzylpiperazine (BZP) are piperazines commonly found in pills sold as Ecstasy. Diphenylprolinol (diphenyl-2-pyrrolidinemethanol [D2PM]) and desoxypipradrol (2-diphenylmethylpiperidine [2-DPMP]) are pipradrol derivatives sold as "legal highs" ("ivory wave") and structurally similar to methylphenidate.

2. Methods

2.1. Chemicals

MDMA, methylphenidate, m-CPP, TFMPP, and BZP were supplied by Lipomed (Arlesheim, Switzerland), and 5-IAI, 2-AI, 2-DPMP, and D2PM were supplied by Cayman Chemicals (Ann Arbor, MI, USA) as racemic hydrochloride salts (purity > 98.5%). MDAI was synthesized as a racemic hydrochloride salt in our laboratory according to Nichols et al. [20]. Radiochemicals (^{3}H-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

The 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 [21] as previously described in detail [22]. 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, or [^{3}H]5-HT (5 nM final concentrations) to initiate the 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 centrifugation at high speed through silicone oil [22]. The uptake times were based on kinetic evaluations showing that uptake is complete after 5 min [22]. 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 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 ratios were calculated as 1/DAT IC_{50}:1/SERT IC_{50}. The DAT/SERT ratio is considered useful to predict the characteristics of the psychoactive effects of novel psychoactive substances [4,23–25]. Higher relative potency at the DAT may indicate a higher abuse potential while relatively increased activity on the 5-HT system is linked to reduced abuse potential and more MDMA-like psychotropic effects [25]. Stimulant amphetamines such as methamphetamine exhibit a DAT/SERT ratio >10, while MDMA and other substances with MDMA-like psychotropic effects exhibit a DAT/SERT ratio close to 0.1 [4,26].

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 [4]. 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 by 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 substrate that diffuses out of the cells and reuptake inhibition [27,28], 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 Dunnett's test to compare test drug-induced monoamine release with nisoxetine, citalopram, and mazindol (negative controls). Compounds that induced significantly higher maximal monoamine efflux compared with the respective transporter inhibitors, which induced slight nonspecific release, were considered monoamine releasers. MDMA was used as a positive control condition in each experiment. Previously published data on cathinones [6] were obtained from the same experiments and tested along-side with the drugs described here. Therefore the data on MDMA are the same as previously published [6] and data on cathinones [6] can be compared with those obtained with the data shown here. All of the conditions were normalized to radioactive counts of the assay buffer control condition. The assays allowed qualitative classification of a drug as a releaser or non-releaser at 100 μM, but not quantitative comparisons between transporters.

2.4. Radioligand binding assays

The radioligand binding assays were performed as described previously [4,22,29]. Briefly, membrane preparations of HEK 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective transporters [21] or receptors (human genes, with the exception of $TAAR_1$ receptors that were rat/mouse; [29]) 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 (DA D_1 receptor), [^{3}H]spiperone and spiperone (DA 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. Similarly obtained data on MDMA has previously been published [4,6].

3. Results

3.1. Monoamine uptake transporter inhibition

The effects of the test compounds on monoamine transporter function are presented in Fig. 2. The corresponding IC_{50} values for monoamine transport inhibition and DAT/SERT inhibition ratios are shown in Table 1. With the exception of m-CPP and TFMPP, all of the tested compounds inhibited NET with IC_{50} values of 0.1–1 μM. For comparison, clinically used NET inhibitors such as reboxetine, indatraline, or duloxetine are slightly more potent and inhibited NET with IC_{50} values of 0.036, 0.43 and 0.126 μM in the same or similar assays [22].

DAT and SERT inhibition potencies varied considerably, resulting in a wide range of DAT/SERT inhibition ratios. Both ring-substituted aminoindanes, 5-IAI and MDAI, and both phenylpiperazines, m-CPP and TFMPP, preferentially inhibited the SERT over the DAT, similar to MDMA [4,6]. The pipradrol derivatives D2PM, 2-DPMP, and methylphenidate were all considerably more potent DAT *vs.* SERT inhibitors. 2-AI and BZP showed only low potency as DAT or SERT inhibitors (IC_{50} values > 10 μM).

3.2. Transporter-mediated monoamine release

The effects of the test compounds on the transporter-mediated release of NE, DA, and 5-HT from transmitter-preloaded cells are depicted in Fig. 3. As expected, MDMA induced significant efflux of NE, DA, and 5-HT compared with the nonspecific "release" observed with the pure uptake inhibitors nisoxetine, mazindol, and citalopram, respectively. The aminoindanes were releasers of at least one monoamine. 5-IAI released 5-HT and DA. MDAI

Table 1
Monoamine uptake transport inhibition.

	NET	DAT	SERT	DAT/SERT ratio
	IC50 [μM] (95% CI)	IC50 [μM] (95% CI)	IC50 [μM] (95% CI)	Ratio (95% CI)
Aminoindans				
5-IAI	0.76 (0.60–0.98)	23 (15–35)	2.5 (1.9–3.4)	0.11
MDAI	0.65 (0.50–0.84)	31 (23–41)	8.3 (3.2–22)	0.2
2-AI	0.54 (0.42–0.69)	58 (4–905)	>100	>1
Piparazines				
m-CPP	1.67 (1.2–2.4)	31 (25–38)	1.2 (0.9–1.6)	0.04
TFMPP	17.5 (8–39)	>100	5.2 (3.8–7.0)	< 0.05
BZP	0.41 (0.33–0.53)	17 (15–19)	57 (40–81)	3.39
Pipradrol derivatives				
D2PM	0.41 (0.34–0.50)	0.86 (0.74–1.0)	38 (4.7–307)	44.36
2-DPMP	0.14 (0.11–0.18)	0.07 (0.06–0.08)	>10	>100
Methylphenidate	0.13 (0.10–0.16)	0.12 (0.09–0.16)	>100	>100

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

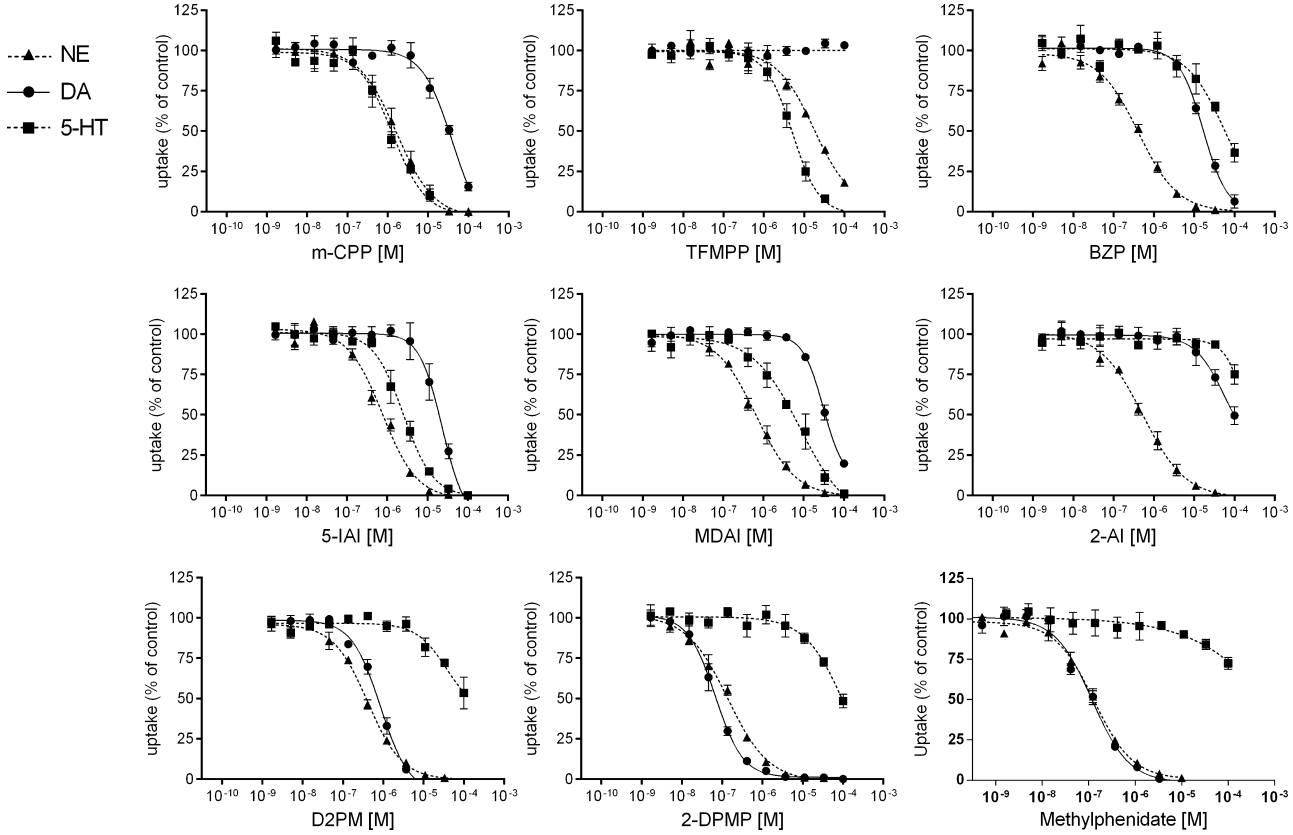

Fig. 2. Monoamine uptake inhibition presented as dose-response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as the mean ± SEM of 3-4 independent experiments. The data were fit by nonlinear regression. The corresponding IC_{50} values are shown in Table 2.

released 5-HT and NE. 2-AI released NE and DA. Among the piperazines, BZP released DA, m-CPP released 5-HT, and TFMPP did not induce the efflux of any monoamine. None of the pipradrol derivatives or methylphenidate was a substrate releaser.

3.3. Binding affinities

Table 2 shows the binding profiles of the test compounds expressed as the potencies of the compounds (K_i) to inhibit radioligand binding to the NET, DAT, and SERT and different monoamine receptors. Among the aminoindanes, the binding profile of MDAI was similar to MDMA [4,6], whereas 5-IAI exhibited submicromolar affinities (<1 μM) for the 5-HT_{1A}, 5-HT_{2A}, α_{2A}, and D_3 receptors. In contrast to MDMA [4,6], the phenylpiperazines m-CPP and TFMPP showed submicromolar (<1 μM) binding to many monoamine receptors, including the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, α_{2A}, and D_{1-3} receptors. The pipradrol derivatives and methylphenidate potently bound to the DAT, but not to any other sites. The aminoindanes, and the phenylpiperazines showed affinity for the rat and mouse $TAAR_1$, similar to MDMA [4,6]. Binding potencies at the monoamine transporters were typically weak, except for the high-affinity (<100 nM) binding of the pipradrol derivatives at the DAT.

4. Discussion

All of the novel substances characterized in the present study interacted with the monoamine transporters. High potency of a compound to inhibit the catecholamine transporter NET and DAT *in vitro* is associated with greater psychostimulant potency in humans [4]. These compounds typically exhibit a DAT/SERT ratio >1 and a high abuse potential [4]. Predominant drug activity at the SERT [22] and a DAT/SERT inhibition ratio of typically 0.01–0.1 are expected to result in subjective drug effects similar to those of MDMA or other empathogens [4,6]. These serotonergic compounds produce subjective well-being and enhanced empathy and sociability in humans without marked psychostimulation [5,30]. Additionally, compounds which predominantly act on SERT and NET [6] have been associated with 5-HT syndrome, hyperthermia and resulting organ failure. Furthermore, compounds which act as monoamine releasers (i.e., MDMA or methamphetamine [4,6]) enter the intracellular space via the transporter. In contrast to pure transporter blockers (i.e., cocaine), monoamine releasers are expected to have more subsequent intracellular pharmacological and neurotoxic consequences [31,32].

The *in vitro* pharmacological profiles of the compounds studied herein may be useful to predict the clinical effects according to the associations noted above. The profiles can also be compared with those of cocaine and a series of recreationally used amphetamine and cathinone derivatives previously characterized using the same *in vitro* assays [4,6].

4.1. Aminoindanes

The aminoindanes 5-IAI and MDAI preferentially inhibited the NET and SERT and less potently inhibited the DAT, similar to MDMA [4,6], but with approximately two-fold lower potency. 5-IAI and MDAI released 5-HT through the SERT, similar to MDMA. MDAI also shared the NE-releasing property and receptor binding profile of MDMA [4,6]. Similar inhibitory effects of 5-IAI and MDAI on human monoamine transporters have recently been shown [7],

Table 2
Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	5-HT$_{1A}$	5-HT$_{2A}$	5-HT$_{2C}$	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	TAAR$_{1rat}$	TAAR$_{1mouse}$
Aminoindanes														
5-IAI	6.3 ± 1.4	5.6 ± 1.5	34 ± 16	0.28 ± 0.08	0.73 ± 0.14	1.2 ± 0.6	>6	0.87 ± 0.33	>12	1.2 ± 0.6	0.68 ± 0.09	7.4 ± 1.3	0.03 ± 0.01	1.1 ± 0.3
MDAI	18 ± 2	12 ± 4	22 ± 12	>17	>12	>12	>6	1.36 ± 0.51	>12	>10	14 ± 2	>13	0.57 ± 0.19	1.8 ± 0.1
2-AI	20 ± 7	21 ± 5	>30	4.0 ± 0.8	>12	>12	>6	0.45 ± 0.10	>12	>10	7.6 ± 2.9	>13	0.31 ± 0.09	2.1 ± 0.4
Piperazines														
m-CPP	3.0 ± 0.4	5.8 ± 1.4	0.63 ± 0.1	0.14 ± 0.01	0.06 ± 0.02	0.13 ± 0.02	0.52 ± 0.01	0.26 ± 0.02	4.0 ± 0.1	2.2 ± 0.8	2.4 ± 0.6	1.5 ± 0.2	0.05 ± 0.01	6.6 ± 1.1
TFMPP	13 ± 2	>25	1.7 ± 0.04	0.17 ± 0.02	0.06 ± 0.01	0.13 ± 0.01	>6	0.73 ± 0.2	>12	1.4 ± 1.0	0.54 ± 0.05	3.3 ± 0.7	0.38 ± 0.06	2.3 ± 0.6
BZP	8.1 ± 0.7	11 ± 4	24 ± 8	> 17	>12	>12	>6	16 ± 5	>12	>10	>16	>13	>10	>10
Pipradrol derivatives														
D2PM	8.2 ± 2.8	0.07 ± 0.03	8.4 ± 1.3	>17	>12	>12	>6	>30	>12	>10	>16	>13	>10	>10
2-DPMP	38 ± 11	0.007 ± 0.001	>30	>17	>12	5.5 ± 0.1	>6	27 ± 9	>12	>10	>16	>13	>10	>10
Methylphenidate	3.3 ± 3.6	0.06 ± 0.01	21 ± 9	NA	>12	NA	>6	20 ± 9	NA	>10	NA	NA	>10	>10

NA, not assessed. Values are K_i given as μM (mean ± SD).

Fig. 3. Monoamine release induced by 100 μM of test compound. 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). 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. In such a batch assay, non-releasing monoamine transporter blockers induce nonspecific "pseudo-efflux" (dashed line, open bars), which arises from substrate that diffuses out of the cells and reuptake inhibition. Only compounds that produced significantly more monoamine efflux ($^*p < 0.05$, $^{***}p < 0.001$) 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 known monoamine releaser MDMA served as a positive control condition for each experiment. The data are expressed as the mean ± SEM of 3–4 independent experiments (with negative and positive controls added in each experiment).

but no comparable data on monoamine release are available. In contrast to the human transporter studies, both MDAI and 5-IAI were relatively more potent SERT and DAT *vs.* NET inhibitors in rat brain synaptosomes [33]. Similar to our data, MDAI released 5-HT, but not DA, and 5-IAI released both 5-HT and DA from rat brain synaptosomes [33]. 5-IAI and MDAI substituted for MDMA in drug discrimination studies [20,34], but were considered less neurotoxic than MDMA [20,34,35]. This profile may increase the popularity of these aminoindanes [13]. The comparable monoamine transporter inhibition and release profile to MDMA [4,6] would predict that MDAI has very similar subjective effects to MDMA, and this is supported by user reports [12,36]. Rare severe complications include serotonin syndrome and hyperthermia [36],

also similar to MDMA. In contrast to MDAI and MDMA [4,6], 5-IAI exhibited relevant binding to 5-HT receptors, including the 5-HT_{2A} receptor that is implicated in the action of hallucinogens [37]. 5-IAI is also considered a less potent MDMA substitute, but dysphoria, anxiety, and hallucinations have also been reported [13]. In contrast to the substituted aminoindanes, 2-AI selectively inhibited the NET, but not the DAT or SERT. This profile is relatively similar to BZP in the present study, but most other amphetamines also typically more potently inhibit the DAT [4,6]. 2-AI also released NE and DA. No comparable data on the pharmacology of 2-AI have been reported. Based on the profile in the present study, 2-AI likely has only mild psychostimulant effects in humans.

4.2. Piperazines

Although piperazines have been widely used since the 1990s, and their pharmacology and toxicology have been reviewed [14,38–41], only few and conflicting original data are available on their pharmacological mechanism. In the present study, BZP inhibited the NET and released DA. Early studies in rats found that BZP inhibits the uptake of not only NE and DA, but also 5-HT [42], which is very inconsistent with our data obtained with human transporters and recent rat studies [43]. Similar to the present study, BZP produced the transporter-mediated release of DA, but not 5-HT from rat synaptosomes *in vitro* [43]. BZP enhanced electrically induced NE release from rabbit arteries [44], likely reflecting its NET-inhibiting properties. BZP also induced a robust increase in extracellular DA *in vivo*, but only weakly increased 5-HT dialysate levels at higher doses [43]. Speculations that BZP may act as an α_2-adrenergic antagonist [44] in humans seem unlikely, given the lack of binding to this and other monoamine receptors in the present study. We also did not confirm the results of an early rat study that reported the 5-HT antagonistic properties of BZP [45]. Thus, our data indicate that BZP is an indirect DA and NE agonist without serotonergic properties. In animals, BZP induced place preference in rats [46] and was self-administered in monkeys, and it substituted for amphetamine in discrimination studies [47]. In humans, 100 mg BZP produced subjective and cardiostimulant effects similar to 7.5–10 mg amphetamine [48,49], consistent with the five- to 10-fold lower potency of BZP at the NET and DAT compared with amphetamine [4]. In healthy women, a dose of 200 mg BZP produced cardiostimulant and subjective effects that were considered similar to those generally seen with stimulants [50], but a direct comparison with other compounds is lacking. The clinical toxicity of BZP mainly includes hallucinations, agitation, seizures, and hyperthermia [40]. Drug users associated more unpleasant effects and hallucinations with BZP than with MDMA [51]. The phenylpiperazines TFMPP and m-CPP preferentially inhibited the SERT as previously reported [52,53]. TFMPP did not act as a 5-HT releaser, and m-CPP only weakly released 5-HT in the present study. SERT-mediated 5-HT release from rat brain synaptosomes or slices has previously been documented for both TFMPP [43,54] and m-CPP [54–56]. Further studies are needed to determine whether the phenylpiperazines differentially interact with the human and rat SERT and whether additional proteins present in the synaptosomal preparations, but not in transfected HEK-293 cells may explain this discrepancy. Also needing clarification is the extent to which the *in vivo* serotonergic action of m-CPP is linked to 5-HT release *vs.* uptake inhibition. In fact, m-CPP has been shown to bind more potently to the SERT than the 5-HT releaser fenfluramine and not to induce long-term 5-HT depletion [53], which are both characteristics of SERT inhibitors rather than 5-HT releasers. m-CPP did not release DA or NE from synaptosomes [56], consistent with our data. Furthermore, we confirmed the previously documented binding of TFMPP and m-CPP to rat 5-HT receptors [52] for the human 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors. In rhesus monkeys, TFMPP has no reinforcing properties and does not maintain responding for amphetamine [47]. Additionally, TFMPP reduced the self-administration of BZP and responding for cocaine [47]. Altogether, the preclinical data indicate that both m-CPP and TFMPP are both indirect and direct serotonergic agonists without relevant dopaminergic activity. However, their precise interaction with the human SERT and the nature of their serotonergic action *in vivo* require further investigations. m-CPP is frequently found in Ecstasy pills as a replacement for MDMA [57,58]. Recreational users consider m-CPP to have less desirable psychotropic effects and more adverse effects, including nausea, compared with MDMA [51,58]. In experimental studies in humans, m-CPP produced mostly dysphoria, weakness, dizziness, anxiety, and nausea [59–61] and less, if any, positive subjective effects, drug liking, and cardiovascular stimulation in direct comparisons with MDMA [62]. The lower clinical potency and efficacy of m-CPP compared with MDMA may be explained by its lower potency as a DAT and NET inhibitor compared with MDMA [4,6] or by its lower efficacy to induce the release of 5-HT. The effects of TFMPP have not been directly compared with other psychoactive substances in humans. TFMPP alone produced moderate dysphoria and amphetamine-type stimulation [63], but not the usual increases in euphoria seen after MDMA administration [64] using the same psychometric scale. Unsurprisingly, therefore, the use of TFMPP alone does not appear to be common [51]. In contrast, BZP in combination with either m-CPP or TFMPP is sometimes sold as Ecstasy [16,41]. Because BZP releases DA, and m-CPP and TFMPP are direct and indirect serotonergic agonists, their combination would be expected to mimic the psychoactive profile of MDMA. In rats, the combination of BZP and TFMPP elevated brain DA and 5-HT levels similarly to MDMA [43]. In humans, the combination of BZP and TFMPP produced stimulation and "good" drug effects, but no euphoria [65]. The BZP-TFMPP combination was not well tolerated at higher doses and frequently produced agitation, anxiety, hallucinations, and vomiting [66], whereas these adverse effects were infrequently observed after MDMA administration in a similar laboratory study [67]. As noted above, the BZP-TFMPP combination has reduced reinforcing properties compared with BZP alone [47], consistent with the abuse-lowering effects of 5-HT.

4.3. Pipradrol derivatives

D2PM and 2-DPMP were selective catecholamine transporter inhibitors without transporter-mediated substrate-releasing properties, similar to methylphenidate. 2-DPMP was a DAT/NET inhibitor that was equally potent to methylphenidate, whereas D2PM was less potent. Consistent with our findings, 2-DPMP has been previously shown to inhibit the human NET and DAT, but not SERT [7], and block the uptake of DA and NE into synaptic rat brain vesicles [68,69]. 2-DPMP also blocked NE uptake into rabbit aortic strips, but did not induce NE release [70], also consistent with our results. Compared with classic stimulants, 2-DPMP was a 10-fold more potent DAT blocker than cocaine [4]. Consistent with the greater DAT-inhibiting potency, 2-DPMP also more potently increased electrically evoked DA release in rat brain slices compared with cocaine [71]. We found no other data on the monoamine uptake and releasing properties of D2PM. The pharmacological profile of the pipradrol derivatives was very similar to the pyrovalerone cathinones MDPV and naphyrone that were characterized in the same assays [4], although naphyrone also inhibits the SERT. MDPV and naphyrone rather than 2-DPMP have been found in some samples of "ivory wave" [72]. Similar to MDPV [4] and naphyrone [73], 2-DPMP and D2PM are highly

lipophilic. Compared with methylphenidate, 2-DPMP lacks polar groups that are typically targeted by metabolic enzymes, resulting in a longer half-life [74,75]. The clinical toxicity of 2-DPMP and D2PM is long-lasting (24–72 h) and involves sympathomimetic stimulation and predominantly psychiatric symptoms, including agitation, hallucinations, and insomnia [17,18]. Altogether, the pipradrol derivatives are potent and selective catecholamine uptake inhibitors, consistent with their potent and prolonged psychostimulant actions. The pharmacological profile is also likely associated with high abuse liability and an increased risk of psychiatric complications.

4.4. $TAAR_1$ binding

The aminoindanes and phenylpiperazines, but not BZP or pipradrol derivatives, exhibited potent $TAAR_1$ binding affinity comparable to MDMA [4,6]. In the present series, all of the serotonergic compounds also bound $TAAR_1$, whereas the affinity for $TAAR_1$ has previously been documented for amphetamine and methamphetamine [4], which only weakly interact with the SERT. Drug activity at the SERT and $TAAR_1$ are both considered to counteract the abuse liability associated with dopaminergic drug properties. Higher serotonergic *vs*. dopaminergic activity has been associated with a lower abuse potential of a drug [4,23–25]. Amphetamines such as MDMA and methamphetamine have been shown to inhibit their own neurochemical and locomotor stimulant effects via $TAAR_1$ activation [76]. The lack of serotonergic activity and lack of $TAAR_1$-mediated "auto-inhibition" in particular with the pipradrol derivatives may contribute to the more stimulant-like and addictive properties of this class of designer compounds compared with classic amphetamines, including MDMA [4].

4.5. Limitations

Knowing the mechanism of action of novel compounds *in vitro* helps to predict potential clinical effects and abuse potential. However, many additional factors also play a role such as brain tissue penetration and pharmacokinetics which need to be further assessed *in vivo*.

5. Conclusion

In summary, the aminoindanes, 5-IAI and MDAI inhibited the SERT and released 5-HT, similar to MDMA [4]. Among the piperazines, BZP interacted with the DAT and NET, and m-CPP and TFMPP interacted with the SERT and serotonergic receptors. The pipradrol derivatives were all potent and selective catecholamine transporter blockers without substrate-releasing properties. The predominant actions of D2PM and 2-DPMP on DAT likely predict a high abuse liability. Further studies are needed to determine potential differences between data obtained with human or rodent transporter studies and to further validate predictions of clinical effects based on such data.

Conflict of interest

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

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Paper 4

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

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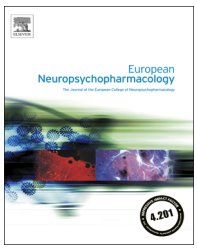

Monoamine transporter and receptor interaction profiles of novel psychoactive substances: Para-halogenated amphetamines and pyrovalerone cathinones

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Transporter

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 the cathinones exhibited reduced trace amine-associated receptor 1 binding compared with the non-β-keto-amphetamines. In conclusion,

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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.

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". Over 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, 2014a, 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, 2014a). In addition, 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 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. Experimental procedures

2.1. Drugs

MDMA, d-amphetamine, d-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 the drugs were obtained as racemic hydrochloride salts, with the exception of amphetamine and methamphetamine, for which the (+)-enantiomer was used and ephedrine, 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, 2014a, 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

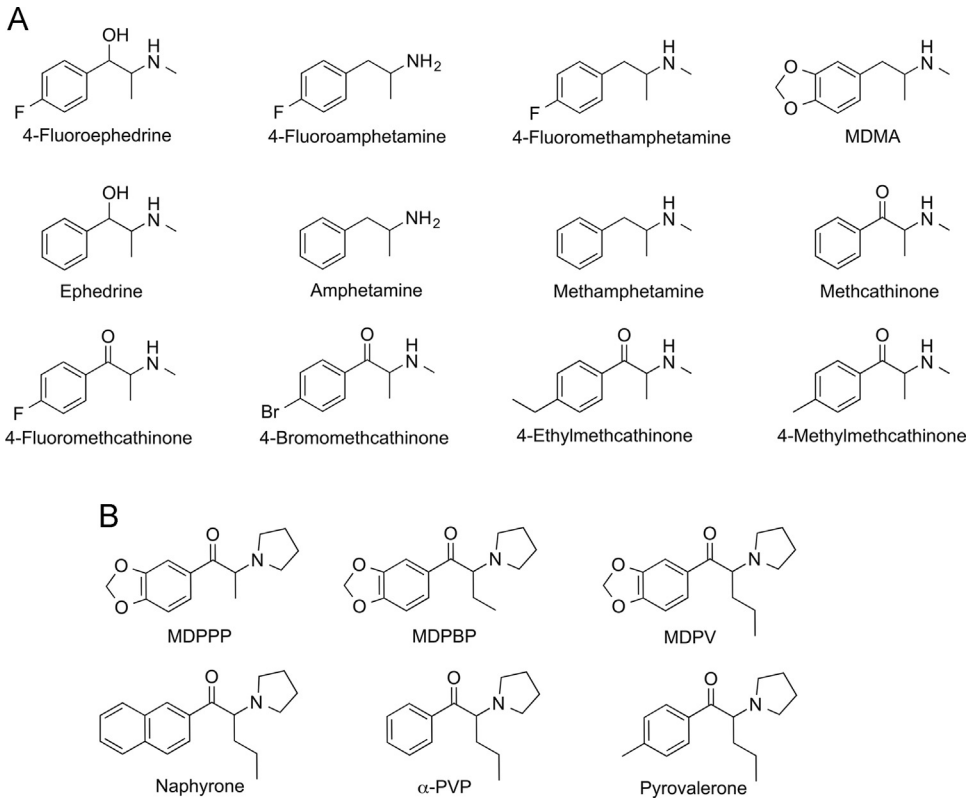

Figure 1 Chemical structures of novel psychoactive substances. A. Para-(4)-substituted amphetamines, 3,4-methylenedioxy-methamphetamine (MDMA, "ecstasy"), and other classic non-para-substituted amphetamines. B. Pyrovalerone-type cathinones.

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)	2.2 (1.6-2.9)	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 IC50 : 1/SERT IC_{50}.

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 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 37 °C 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 31 °C. 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 31 °C. 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 at 37°C 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 in Figure 2 and 3, and the corresponding IC_{50} values and DAT:SERT inhibition ratios are listed in Table 1. In all cases, the para-(4) substitution (Figure 1A) reduced the potency of the amphetamines to inhibit both 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 and Figure 2). 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; Figure 2). In contrast, all of the pyrovalerone cathinones (Figure 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 and Figure 3). One exception was naphyrone, which also inhibited the SERT at submicromolar concentrations. The 3,

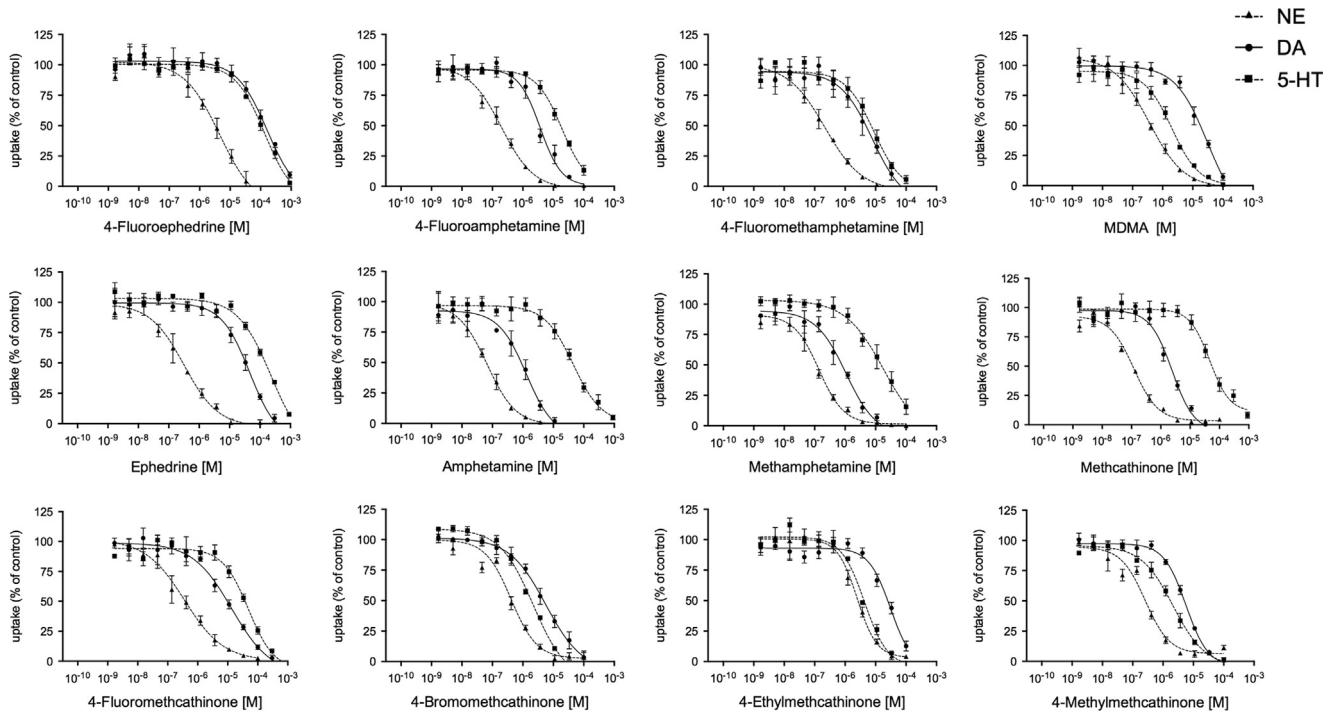

Figure 2 Effects of para-(4)-substituted and non-substituted amphetamines on monoamine transport. Monoamine uptake inhibition is presented as concentration-response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The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

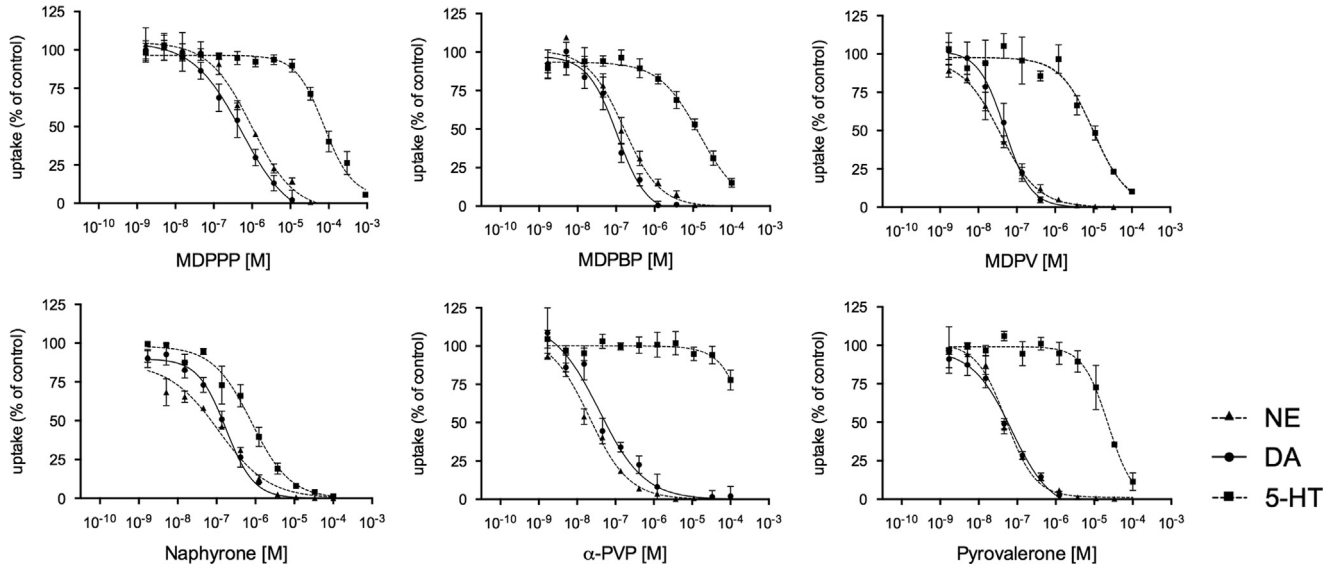

Figure 3 Effects of pyrovalerone cathinones on monoamine transport. Monoamine uptake inhibition is presented as concentration-response curves for the inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as the mean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

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 (Figure 1B) did not appear to interact with the SERT at submicromolar concentrations, with the exception of naphyrone (Figure 3).

3.2. Transporter-mediated monoamine release

Monoamine release is shown in Figure 4. All the para-substituted amphetamine derivatives released NE and DA similarly to their non-substituted classic analogs amphetamine, methamphetamine, and methcathinone. In addition, 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 (Figure 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 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. In addition, 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 NET and DAT, confirming previous studies with MDPV (Baumann et al.,

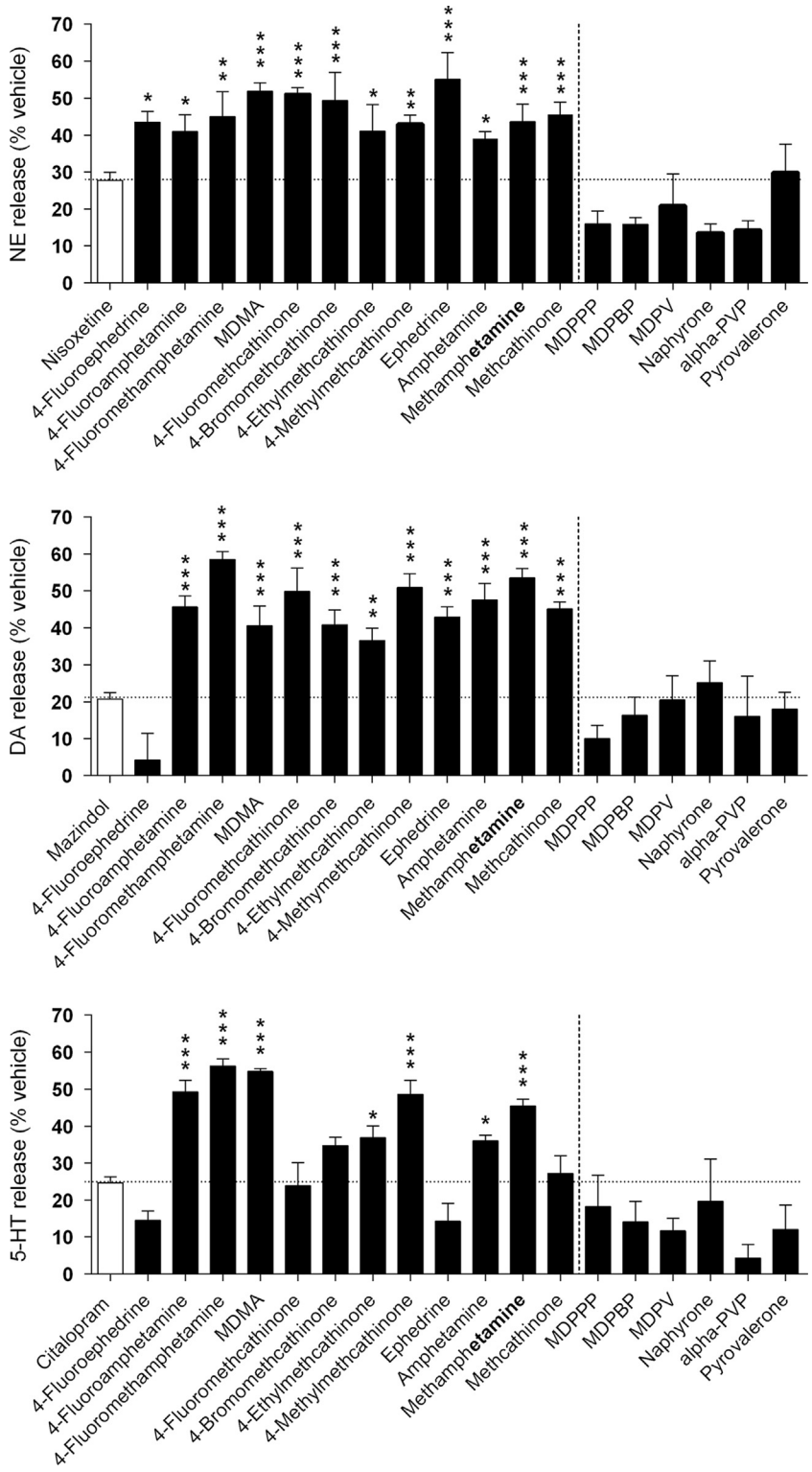

Figure 4 Effect of all substances on monoamine release. HEK 293 cells that expressed NET, DAT, and SERT were loaded with [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT, respectively, washed, and incubated with a high concentration of the compounds (100 μM). All para-substituted and non-substituted amphetamines 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 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 2 Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$	$TAAR_{1human}$
Para-(4)-substituted amphetamines												
4-Fluoroephedrine	17.6±2.4	27.7±15	39.1±11	> 4.9	8.4±1.2	> 12	> 20	> 17	> 13	2.6±1.2	17.6±8.3	> 20
4-Fluoroamphetamine	13.5±1.5	11.0±4.2	32.1±9.4	> 4.9	4.4±0.3	> 12	> 20	> 17	> 13	0.08±0.04	0.32±0.10	2.3±1.9
4-Fluoromethamphetamine	9.0±0.6	10.8±1.4	35±12	> 4.9	2.6±0.3	> 12	> 20	> 17	7.1±1.6	0.24±0.1	1.7±0.9	6.5±4.4
MDMA[b]	26.8±8.7	8.4±3.3	13.0±2.3	> 6	15.0±10	> 12	25±13	> 17	> 13	0.37±0.1	2.4±1.1	14.6±1.8
4-Fluoromethcathinone[a]	> 25	12.2±3.1	> 30	1.5±0.1	> 20	> 12	> 30	> 17	> 13	5.4±1.7	> 10	> 20
4-Bromomethcathinone	6.5±1.4	3.6±0.3	8.3±2.2	8.2±3.0	12.7±0.2	> 12	>10	> 17	2.1±0.1	1.8±0.1	12.9±2.7	> 20
4-Ethylmethcathinone	16.2±2.2	28±16	17.5±3.6	8.4±3.4	21.1±7.6	> 12	>10	> 17	> 13	> 20	> 20	> 20
4-Methylmethcathinone[a]	> 25	3.4±0.8	> 30	3.5±2.2	11.0±5.0	> 12	> 30	> 9	> 13	4.3±2.0	> 10	> 20
Non para-(4)-substituted amphetamines												
Ephedrine	> 30	> 30	> 30	> 12	4.1±0.5	> 12	> 25	> 17	> 13	3.7±0.9	> 15	17.1±4.1
Amphetamine[a]	1.0±0.6	5.7±3.8	> 25	> 6	2.8±0.8	> 12	> 30	> 17	> 13	0.23±0.2	0.09±0.06	0.22±0.13
Methamphetamine[b]	3.0±2.2	1.8±0.7	24.6±10	> 6	6.1±1.6	> 12	> 30	> 17	> 13	0.35±0.1	0.55±0.24	1.4±0.5
Methcathinone[b]	1.4±0.7	1.3±0.2	> 30	3.9±1.3	11.9±3.9	> 12	> 30	> 9	> 13	4.1±1.2	> 10	> 20
Pyrovalerone cathinones												
MDPBP	1.1±0.1	0.02±0.002	4.1±1.2	> 4.9	9.4±1.6	> 12	> 20	> 17	> 13	> 20	> 20	> 20
MDPPP	3.5±1.0	0.18±0.05	11.7±1.0	> 15	13.9±0.9	> 12	> 10	> 17	8.7±0.6	16.1±6.7	> 20	> 20
MDPV[a]	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[a]	0.18±0.02	0.04±0.01	0.18±0.02	> 6	7.9±2.8	> 12	> 20	> 17	2.3±0.3	> 20	> 20	> 20
α-PVP	0.06±0.02	0.007±0.002	> 30	> 15	> 20	> 12	> 10	> 17	> 13	16.3±6.4	> 20	> 20
Pyrovalerone[a]	0.06±0.01	0.03±0.005	5.0±0.3	> 6	> 20	> 12	> 30	> 9	10.7±1.5	> 12	> 10	> 20

Values are K_i given as μM (mean ±SD).
[a]Values are from Simmler et al. (2013) except for the $TAAR_1$ human binding.
[b]Values are from Simmler et al. (2014a, 2014b) except for the $TAAR_1$ human binding.

Table 3 Serotonin 5-HT2A and 5-HT2C receptor binding affinities and functional 5-HT2B receptor activity.

	5-HT_{1A}	5-HT_{2A}	5-HT_{2B}		5-HT_{2C}
	Receptor binding $K_i \pm$ SEM (μM)	Receptor binding $K_i \pm$ SEM (μM)	Activation potency $EC_{50} \pm$ SEM (μM)	Activation efficacy % maximum ± SEM	Receptor binding $K_i \pm$ SEM (μM)
Para-(4)-substituted amphetamines					
4-Fluoroephedrine	> 17	> 13	> 20	0	3.7 ± 1.1
4-Fluoroamphetamine	4.4 ± 0.8	11.3 ± 2.6	11.4 ± 4.6	49 ± 15	7.8 ± 0.7
4-Fluoromethamphetamine	5.0 ± 1.9	3.8 ± 0.7	> 20	0	5.5 ± 0.6
MDMA[b]	12.2 ± 0.8	5.9 ± 2.7	> 20	0	> 13
4-Fluoromethcathinone[a]	> 20	1.4 ± 0.6	> 20	0	> 13
4-Bromomethcathinone	> 20	3.2 ± 0.6	> 20	0	>13
4-Ethylmethcathinone	> 20	6.5 ± 0.9	> 20	0	9.6 ± 0.4
4-Methylmethcathinone[a]	> 20	2.1 ± 0.7	> 20	0	> 13
Non para-(4)-substituted amphetamines					
Ephedrine	> 20	> 13	> 20	0	3.3 ± 0.7
Amphetamine[a]	6.7 ± 1.4	> 13	9.4 ± 1.6	8 ± 2	> 13
Methamphetamine[a]	8.1 ± 0.7	> 13	> 20	0	> 13
Methcathinone[a]	12.8 ± 3.5	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[a]	10.3 ± 4.7	> 13	> 20	0	> 13
Naphyrone[a]	6.0 ± 0.2	11.7 ± 2.2	>20	0	> 13
α-PVP	5.2 ± 0.1	> 13	> 20	0	> 13
Pyrovalerone[a]	13.4 ± 2.1	> 13	> 20	0	> 13

[a]Binding values are from Simmler et al. (2013) and are included for comparison.
[b]Binding values are from Simmler et al. (2014a, 2014b) and are included for comparison.

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). In addition, 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). In addition, 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. In addition, 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, 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. In addition, 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.

Contributors

AR, MCH and MEL designed the study. MEL and MCH obtained funding. AR, MCH and MEL conducted experiments, analyzed data and wrote the manuscript. All the authors reviewed and approved the manuscript.

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Paper 5

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RESEARCH PAPER

Pharmacological profile of novel psychoactive benzofurans

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BACKGROUND AND PURPOSE

Benzofurans are newly used psychoactive substances, but their pharmacology is unknown. The aim of the present study was to pharmacologically characterize benzofurans *in vitro*.

EXPERIMENTAL APPROACH

We assessed the effects of the benzofurans 5-APB, 5-APDB, 6-APB, 6-APDB, 4-APB, 7-APB, 5-EAPB and 5-MAPDB and benzodifuran 2C-B-FLY on the human noradrenaline (NA), dopamine and 5-HT uptake transporters using HEK 293 cells that express the respective transporters. We also investigated the release of NA, dopamine and 5-HT from monoamine-preloaded cells, monoamine receptor-binding affinity and 5-HT_{2A} and 5-HT_{2B} receptor activation.

KEY RESULTS

All of the benzofurans inhibited NA and 5-HT uptake more than dopamine uptake, similar to methylenedioxymethamphetamine (MDMA) and unlike methamphetamine. All of the benzofurans also released monoamines and interacted with trace amine-associated receptor 1 (TA_1 receptor), similar to classic amphetamines. Most benzofurans were partial 5-HT_{2A} receptor agonists similar to MDMA, but also 5-HT_{2B} receptor agonists, unlike MDMA and methamphetamine. The benzodifuran 2C-B-FLY very potently interacted with 5-HT_2 receptors and also bound to TA_1 receptors.

CONCLUSIONS AND IMPLICATIONS

Despite very similar structures, differences were found in the pharmacological profiles of different benzofurans and compared with their amphetamine analogues. Benzofurans acted as indirect monoamine agonists that interact with transporters similarly to MDMA. The benzofurans also interacted with 5-HT receptors. This pharmacological profile probably results in MDMA-like entactogenic psychoactive properties. However, benzofurans induce 5-HT_{2B} receptor activation associated with heart valve fibrosis. The pharmacology of 2C-B-FLY indicates predominant hallucinogenic properties and a risk for vasoconstriction.

Abbreviations

2C-B-FLY, 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine; 4-APB, 4-(2-aminopropyl)benzofuran; 5-APB, 5-(2-aminopropyl)benzofuran; 5-APDB, 5-(2-aminopropyl)-2,3-dihydrobenzofuran; 5-EAPB, 5-(2-ethylaminopropyl) benzofuran; 5-MAPDB, 1-(2,3-dihydrobenzofuran-5-yl)-N-methylpropan-2-amine; 6-APB, 6-(2-aminopropyl)benzofuran; 6-APDB, 6-(2-aminopropyl)-2,3-dihydrobenzofuran; 7-APB, 7-(2-aminopropyl)benzofuran; β-keto-MDA, β-keto-3,4-methylenedioxyamphetamine; bromo-dragonFLY, 1-(8-bromobenzo[1,2-b;4,5-b′]difuran-4-yl)-2-aminopropane; DAT, dopamine transporter; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; NET, noradrenaline transporter; SERT, 5-HT transporter; TA receptor, trace amine-associated receptor

Tables of Links

TARGETS		
GPCRs[a]		**Transporters**[b]
5-HT1A receptor	D1 receptor	DAT
5-HT2A receptor	D2 receptor	NET
5-HT2B receptor	D3 receptor	SERT
5-HT2C receptor	H1 receptor	
α1A adrenoceptor	TA1 receptor	
α2A adrenoceptor		

LIGANDS		
5-HT	Noradrenaline	Pyrilamine
Butaclamol	Mazindole	Rauwolscine
Citalopram	MDMA	Risperidone
Clozapine	Mesulergine	RO5166017
Dopamine	Methamphetamine	SCH23390
Ketanserin	Mianserin	Spiperone
LSD	Phentolamine	WIN35428
Nisoxetine	Prazosin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Novel psychoactive substances are newly used designer drugs ('Internet drugs', 'research chemicals', 'legal highs') that potentially pose similar health risks to classic illicit substances. In recent years, the number of newly detected psychoactive substances on the illicit drug market has dramatically increased. In the European Union, 41 novel psychoactive substances were identified for the first time in 2010, 49 were identified in 2011, 73 were identified in 2012 and 81 were identified in 2013 within the European Early Warning System (EMCDDA, 2014).

Benzofurans are a group of novel psychoactive substances (King, 2014) of particular interest because they are structurally very similar to the popular recreational drug 3,4-methylenedioxymethamphetamine (MDMA) and its active metabolite 3,4-methylenedioxyamphetamine (MDA; Greene, 2013). 5-(2-Aminopropyl)benzofuran (5-APB) and 6-(2-aminopropyl)benzofuran) (6-APB) are benzofuran analogues of MDA (Figure 1). 5-(2-Aminopropyl)-2,3-dihydrobenzofuran (5-APDB) and 6-(2-aminopropyl)-2,3-dihydrobenzofuran (6-APDB) are dihydrobenzofuran analogues (Figure 1) that were originally synthesized for research purposes (Monte *et al.*, 1993). 4-(2-

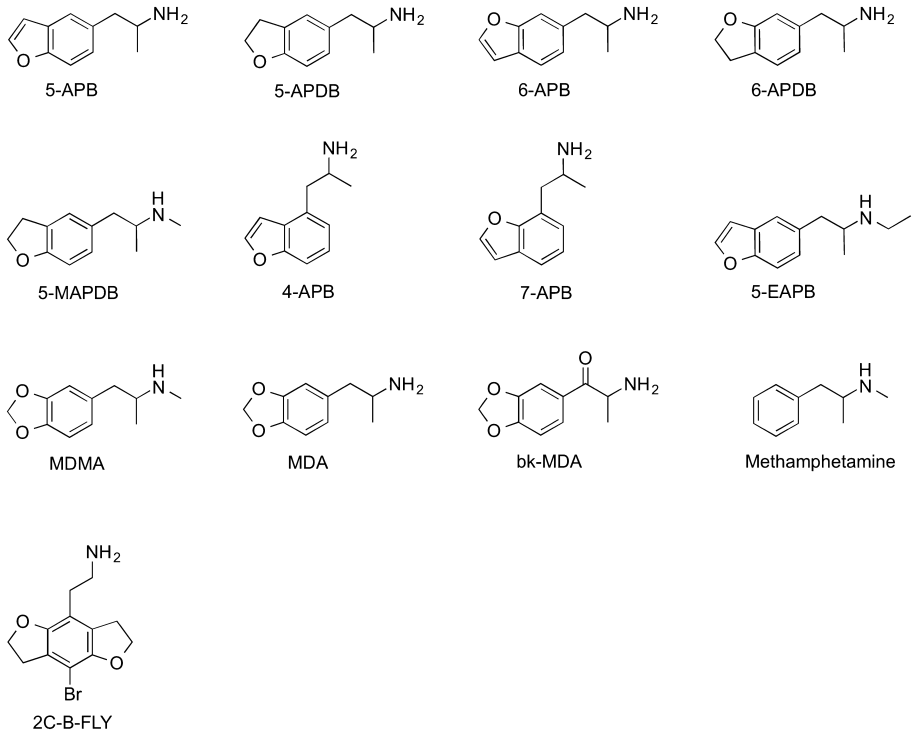

Figure 1

Chemical structures of benzofurans and related amphetamines.

Aminopropyl)benzofuran (4-APB) and 7-(2-aminopropyl)benzofuran (7-APB) are positional isomers of 5-APB and 6-APB. 1-(2,3-Dihydrobenzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPDB) is a dihydrobenzofuran analogue of MDMA, and 5-(2-ethylaminopropyl)benzofuran (5-EAPB) is a benzofuran analogue of MDMA but with an *N*-ethyl group (Figure 1).

5-APB and 6-APB appeared on the drug market in 2010–2011 (Chan *et al.*, 2013; Jebadurai *et al.*, 2013; Stanczuk *et al.*, 2013; Archer *et al.*, 2014; Elliott and Evans, 2014; King, 2014), with reports of intoxication (Chan *et al.*, 2013; Greene, 2013; Jebadurai *et al.*, 2013; Seetohul and Pounder, 2013). 4-APB was first reported to the EMCDDA in 2010 (King, 2014) and is typically detected in products that are sold as 6-APB as a by-product (Stanczuk *et al.*, 2013; Strano Rossi *et al.*, 2014). Users report that the effects of 5-APB and 6-APB are comparable with MDMA but more intense (Greene, 2013; Jebadurai *et al.*, 2013). Adverse effects include nausea, sympathomimetic stimulation and agitation (Chan *et al.*, 2013; Greene, 2013). 5-APDB and 6-APDB were first reported to the EMCDDA in 2012, and another three benzofurans, including 5-EAPB, were first reported in 2013 (King, 2014). Presently, no published studies have reported the psychotropic and toxic effects of these benzofurans, but 5-APDB, 6-APDB and 5-EAPB are being discussed in drug user forums (Bluelight, 2013a,b; Drugs-Forum, 2013). Little is known about the pharmacology of benzofurans. 5-APB and 6-APB have been shown to inhibit the human dopamine, noradrenaline and 5-HT transporters (DAT, NET and SERT, respectively; Iversen *et al.*, 2013) and are agonists at the rat 5-HT_{2A} receptor (Dawson *et al.*, 2014) and human and rat 5-HT_{2B} receptor (Iversen *et al.*, 2013; Dawson *et al.*, 2014). Additionally, fast cyclic voltammetry experiments in rat brain slices indicated that 5-APB releases dopamine at high concentrations (Dawson *et al.*, 2014). 5-APDB and 6-APDB also inhibited the monoamine transporters with greater affinity for the SERT over the DAT compared with MDA in crude rat synaptosome preparations (Monte *et al.*, 1993).

The benzodifurans 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine (2C-B-FLY) and 1-(8-bromobenzo[1,2-b;4,5-b′]difuran-4-yl)-2-aminopropane) (bromo-dragonFLY) are known as 'fly' drugs because of their chemical structures (Figure 1). A series of benzodifurans were originally synthesized to study 5-HT_{2A} receptor function (Monte *et al.*, 1997; Parker *et al.*, 1998; Chambers *et al.*, 2001). The recreational use of 2C-B-FLY and bromo-dragonFLY began to be reported in 2007 (Andreasen *et al.*, 2009; Greene, 2013; King, 2014), and there are case reports of severe agitation, hallucinations, seizures and fatalities associated with bromo-dragonFLY (Andreasen *et al.*, 2009; Wood *et al.*, 2009; Nielsen *et al.*, 2010). 2C-B-FLY and bromo-dragonFLY are potent 5-HT_{2A} receptor agonists (Monte *et al.*, 1996; Chambers *et al.*, 2001), but interactions with other monoamine receptors and their transporters have not been tested.

Systematic evaluations of the pharmacological profiles of benzofurans are lacking. We determined the potencies of a series of benzofurans and the benzodifuran 2C-B-FLY to inhibit the DAT, NET and SERT and tested transporter-mediated monoamine release *in vitro*. We also characterized the binding profiles at monoamine receptors and assessed 5-HT_{2A} and 5-HT_{2B} receptor activation. The 5-HT_{2A} receptor mediates hallucinogenic effects (Nichols, 2004), and the 5-HT_{2B} receptor has been implicated in drug-associated endocardial fibrosis (Roth, 2007). MDMA, MDA, β-keto-MDA and methamphetamine were included as comparator substances.

Methods

Monoamine uptake transport inhibition

Inhibition of the human NET, DAT and SERT was assessed in HEK 293 cells that were stably transfected with the transporters as specified previously (Hysek *et al.*, 2012c). Briefly, the cells were suspended in uptake buffer. We incubated the cells for 10 min with different concentrations of the test compounds and then added the corresponding [^{3}H] monoamine (5 nM final concentration) at room temperature. After 10 min, we stopped uptake by separating the cells from the buffer using centrifugation through silicone oil (Hysek *et al.*, 2012c). 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 β-counter. Non-specific 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%). The data were fitted by non-linear regression to variable slope sigmoidal dose–response curves, and IC_{50} values were calculated using Prism software (GraphPad, San Diego, CA, USA). DAT : SERT inhibition ratios were calculated as 1/DAT IC_{50}:1/SERT IC_{50}. Higher relative potency at the DAT indicates a higher abuse potential, whereas relatively increased activity of the 5-HT system is linked to a reduction in 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; 2014a,b).

Transporter-mediated monoamine release

We studied the effects of a single high dose (100 μM) of the test compounds on transporter-mediated NA, 5-HT and dopamine efflux in HEK 293 cells that overexpressed the respective human monoamine transporter, as previously reported in detail (Simmler *et al.*, 2013). Briefly, adherent cells were incubated with the respective radiolabeled monoamine (10 nM [^{3}H]-NA and 10 μM unlabelled NA, 10 nM [^{3}H]-dopamine and 1 μM unlabelled dopamine, and 10 nM [^{3}H]-5-HT) for 20 min at 37°C. We then washed the cells twice with buffer and added 1 mL of buffer that contained the test compound (100 μM final concentration). We stopped [^{3}H]-5-HT and [^{3}H]-dopamine release after 15 min and [^{3}H]-NA release after 45 min by washing twice with ice-cold buffer. We quantified the radioactivity that remained in the cells. Non-specific 'pseudo-efflux', which arises from non-specific 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.

ANOVA followed by the Holm–Sidak test was used to compare compound-induced release with the negative controls. Substances that induced significantly higher monoamine efflux compared with the negative control were considered monoamine releasers.

Radioligand binding assays

The radioligand binding assays were performed as described previously (Hysek *et al.*, 2012c; 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, except rat and mouse genes for TA_1 receptor; 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 non-specific 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 adrenoceptor), [^{3}H]-rauwolscine and phentolamine (α_2 adrenoceptor), [^{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 (histamine H_1 receptor), and [^{3}H]-RO5166017 and RO5166017 (TA_1 receptor). IC_{50} values were determined by calculating non-linear 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.

Functional 5-HT_{2A} and 5-HT_{2B} receptor activity

The 5-HT_{2B} receptor functional assay was performed as described previously (Jensen *et al.*, 2008). Briefly, human 5-HT_{2B} receptor-expressing HEK 293 cells were incubated at 37°C 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, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31°C. The Fluo-4 solution was removed by snap inversion, and 100 µL of Fluo-4 solution was added a second time. The cells were then incubated for another 45 min at 31°C. Immediately before testing, the cells were washed with HBSS (Gibco) and 20 mM HEPES (assay buffer; Gibco, Life Technologies, Zug, Switzerland) 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 online. The increase in fluorescence was then measured. EC_{50} values were derived from the concentration–response curves using non-linear regression. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

Cytotoxicity

To confirm cell integrity during the pharmacological assays, cytotoxicity was assessed using the ToxiLight™ bioassay (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The assay quantitatively measures the release of adenylate kinase from damaged cells providing a highly sensitive method for measuring cytolysis (Crouch *et al.*, 1993; Hysek *et al.*, 2012c; Felser *et al.*, 2014). Cells grown in 96-well plates were exposed to the compounds at a high concentration of 100 µM. All test conditions contained DMSO 0.1% (v:v) which is non-toxic and was also used as negative control. Triton™ X-100 (0.1%, Sigma-Aldrich, Buchs, Switzerland) lyses cells and was used as positive control. After 4 h of incubation at 37°C, 10 µL of supernatant per well was removed and combined with 50 µL of ToxiLight™ reagent and luminescence recorded using a Tecan Infinite™ 200 Pro (Tecan, Männedorf, Switzerland) plate reader.

Statistical analyses

The uptake transporter inhibition data were fit by non-linear regression to variable-slope sigmoidal dose-response curves, and IC_{50} values were calculated using Prism software (GraphPad, San Diego, CA, USA). ANOVA followed by the Holm-Sidak test was used to compare compound-induced release with the negative controls. Substances that induced significantly higher monoamine efflux compared with the negative control were considered monoamine releasers. IC_{50} values for radioligand binding 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. EC_{50} values for 5-HT_2 receptor activation were derived from the concentration-response curves using nonlinear regression.

Drugs

MDMA, MDA, β-keto-MDA, methamphetamine and 2C-B-FLY were obtained from Lipomed (Arlesheim, Switzerland). 6-APB, 6-APDB, 5-APB, 5-APDB, 4-APB, 7-APB and 5-MAPDB were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 5-EAPB was obtained from the Forensic Institute (Zurich, Switzerland). All of the drugs were used as racemic hydrochloride salts, with the exception of d-methamphetamine. Purity was at least 98% for all of the substances, with the exception of 2C-B-FLY, whose purity was approximately 95% as determined by HPLC.

Results

Monoamine uptake transporter inhibition

Uptake inhibition curves are depicted in Figure 2, and the corresponding IC_{50} values and DAT : SERT inhibition ratios are listed in Table 1. All of the benzofurans inhibited the NET at submicromolar concentrations, similar to MDMA, MDA and methamphetamine. All of the benzofurans were weak DAT inhibitors compared with methamphetamine and more similar to MDMA, which was also a weak DAT inhibitor. Only

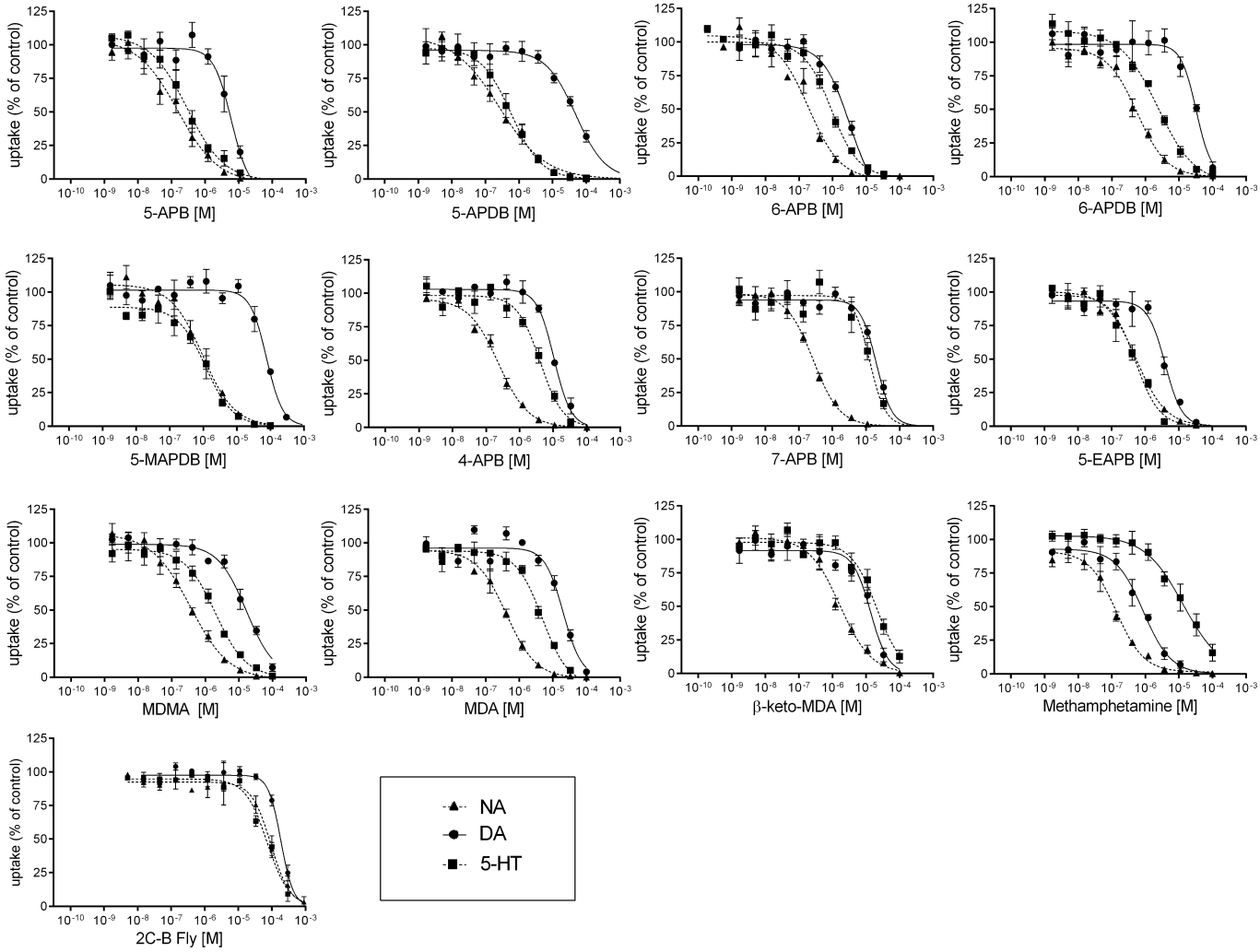

Figure 2

Monoamine uptake transporter inhibition. Concentration–response curves show the uptake inhibition of [^{3}H]-NA, [^{3}H]-dopamine and [^{3}H]-5-HT in HEK 293 cells transfected with the respective monoamine transporter. The data are expressed as the mean ± SEM of three to four independents experiments. Curves were fitted to the data with non-linear regression. The corresponding IC_{50} values are shown in Table 1.

5-APB, 6-APB and 5-EAPB were more potent at the DAT compared with MDMA and MDA. In contrast, the dihydrobenzofurans 5-APDB, 6-APDB and 5-MAPDB were inactive at the DAT (IC_{50} >30 μM). 5-APB, 5-APDB, 6-APB and 5-EAPB inhibited the SERT at submicromolar concentrations and more potently than MDMA. 6-APDB and 5-MAPDB inhibited the SERT in the 1–3 micromolar concentration range, similar to MDMA. 4-APB and 7-APB exhibited low potency at the SERT, more similar to methamphetamine. The DAT : SERT inhibition ratio for all of the benzofurans was low, consistent with greater 5-hydroxytryptaminergic versus dopaminergic activity that is overall similar to MDMA. The dihydrobenzofurans (5-APDB, 6-APDB and 5-MAPDB) and 5-APB exhibited the lowest DAT : SERT inhibition ratios (lower than MDMA). In contrast, 4-APB and 7-APB exhibited the highest DAT : SERT inhibition ratios, consistent with their low potency at the SERT and showing a profile that is between MDMA and methamphetamine with regard to 5-hydroxytryptaminergic versus dopaminergic activity. In terms of structure–activity relationships, the dihydro-compounds 5-APDB and 6-APDB had similar noradrenergic and 5-hydroxytryptaminergic activities compared with their analogues 5-APB and 6-APB but were markedly less potent at the DAT. The monoamine transporter inhibition potencies of the positional isomers 4-APB and 7-APB were reduced, particularly for the SERT, compared with their analogues 5-APB and 6-APB. Additionally, the oxygen in the para-position for 5-APB and 5-APDB resulted in higher absolute and relative potency at the SERT compared with 6-APB and 6-APDB respectively. β-Keto-substitution in the β-keto-MDA versus MDA structures increased dopaminergic versus 5-hydroxytryptaminergic activity. The benzodifuran 2C-B-FLY was inactive at all of the monoamine transporters (IC_{50} >50 μM).

Monoamine release

At high concentrations, all of the benzofurans released at least one of the monoamines through the respective

Table 1

Monoamine transporter inhibition

	NET	DAT	SERT	DAT/SERT inhibition ratio
	IC_{50} (μM) (95% CI)	IC_{50} (μM) (95% CI)	IC_{50} (μM) (95% CI)	Ratio (95% CI)
Benzofurans				
5-APB	0.16 (0.08–0.3)	6.1 (4–9)	0.29 (0.17–0.5)	0.05 (0.02–1.2)
5-APDB	0.29 (0.2–0.5)	49 (33–73)	0.58 (0.4–0.9)	0.01 (0.005–0.03)
6-APB	0.19 (0.1–0.3)	3.3 (2.4–4.5)	0.93 (0.7–1.3)	0.29 (0.16–0.54)
6-APDB	0.56 (0.4–0.8)	33 (25–43)	2.3 (1.4–3.9)	0.07 (0.03–0.16)
5-MAPDB	0.96 (0.5–1.7)	77 (62–96)	1.2 (0.7–2)	0.02 (0.01–0.03)
4-APB	0.24 (0.2–0.3)	12 (9–16)	5.5 (3.4–8.7)	0.46 (0.21–1.0)
7-APB	0.27 (0.2–0.3)	20 (16–26)	13 (9–18)	0.65 (0.35–1.1)
5-EAPB	0.56 (0.4–0.7)	4.9 (3–8)	0.72 (0.5–1.1)	0.15 (0.07–0.35)
Benzodifuran				
2C-B-FLY	94 (72–124)	187 (161–217)	73 (58–92)	0.39 (0.27–0.57)
Related amphetamines				
MDMA	0.36 (0.2–0.6)	16.7 (16.3–17)	2.4 (1.4–3.0)	0.14 (0.08–0.18)
MDA	0.42 (0.3–0.6)	20.5 (20.3–20.6)	4.9 (3.5–6.8)	0.24 (0.17–0.33)
β-Keto-MDA	1.6 (1.1–2.3)	14 (10–18)	21 (15–28)	1.5 (0.8–2.8)
Methamphetamine	0.14 (0.09–0.2)	0.87 (0.84–0.91)	13.6 (13.5–13.8)	15.6 (14.8–16.4)

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}.

monoamine transporter, similar to the amphetamines (Figure 3). In contrast, 2C-B-FLY was not a monoamine releaser.

Binding affinities

The benzofurans interacted with the monoamine transporters but also with several monoamine receptors (Tables 2 and 3). All of the benzofurans exhibited submicromolar affinity for the TA_1 receptor, except for 5-EAPB, which was inactive at mouse TA_1 receptos. Benzofurans showed mostly higher potency at TA_1 receptors than the classic amphetamines. All of the benzomonofurans exhibited binding affinities for the 5-HT_{2A} receptor in the micromolar range (0.8–3.4 μM). Functionally, most of them acted as low-potency partial agonists similar to MDMA and MDA but unlike methamphetamine. Most of the benzofurans were also partial agonists at the 5-HT_{2B} receptor. In contrast MDMA and methamphetamine did not stimulate 5-HT_{2B} receptors. With the exception of 7-APB and 5-EAPB, the benzofurans exhibited submicromolar binding affinities at the 5-HT_{2C} receptor. Binding potencies at the 5-HT_1 receptor varied among different benzofurans. Only 7-APB showed submicromolar binding affinity. Potent binding to most of the assessed 5-HT receptor subtypes distinctly discriminated the benzofurans from the pharmacological profiles of their related amphetamines, which exhibited no or low 5-HT_{1A} affinity and did not bind to 5-HT_{2B} or 5-HT_{2C} receptors except for MDA with a K_i value of 3 μM at 5-HT_{2C}. Most of the benzofurans bound to α_{1A}- and α_{2A}-adrenoceptors in the 3–12 and 0.1–6 μM ranges respectively. There was no binding to dopamine receptors and only low-affinity binding to histamine H_1 receptors (>10 μM for most of the drugs). The benzodifuran 2C-B-FLY did not bind to the monoamine transporters but interacted with all of the receptors tested in the present study and particularly exhibited high affinity for TA_1 receptors and all of the 5-HT_2 receptors. Importantly, 2C-B-FLY was a very potent agonist at the 5-HT_{2A} receptor. 2C-B-FLY thus exhibited a pharmacological profile that was distinct from the mono-benzofurans and related amphetamines.

Cytotoxicity

None of the compounds investigated produced cytotoxicity, thus confirming cell integrity during the functional assays in this study.

Discussion

We determined the *in vitro* pharmacological profiles of new benzofurans that are recreationally abused compared with their well-known amphetamine analogues. The benzofurans blocked monoamine transporters and induced transporter-mediated monoamine release similarly to MDMA. More than MDMA and methamphetamine, the benzofurans also directly stimulated adrenoceptors and 5-HT receptors. The benzodifuran 2C-B-FLY was a potent agonist at 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, consistent with the reported hallucinogenic properties of 2C-B-FLY.

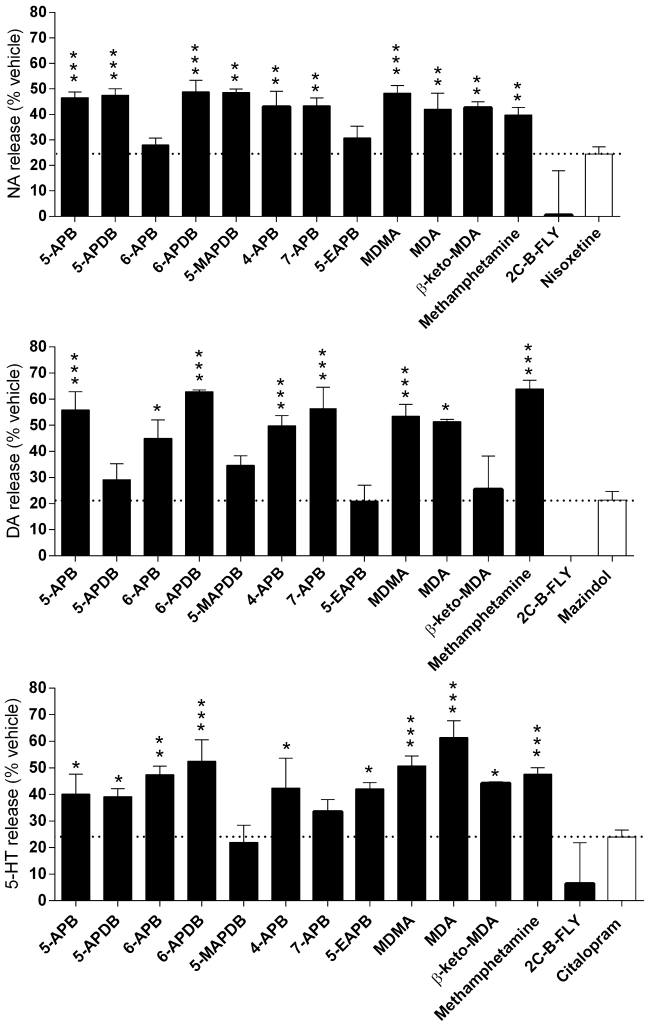

Figure 3

Monoamine release. Monoamine release was induced by a high concentration of the compound (100 μM) after preloading the transporter-transfected cells with the respective radiolabelled monoamine. All of the benzofurans released NA, dopamine and 5-HT similarly to methamphetamine and MDMA. In contrast, the benzodifuran 2CB-FLY was not a monoamine releaser. Transporter blockers induced non-specific '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 respective non-releasing uptake inhibitors (negative controls, open bars) were considered monoamine releasers. The data are expressed as the mean ± SEM of three to four independent experiments.

Monoamine uptake transporter inhibition and monoamine release

All of the benzofurans inhibited the NET at submicromolar concentrations, similar to MDMA, MDA and methamphetamine. NA mediates sympathomimetic stimulation (Hysek *et al.*, 2011), and this finding predicts the cardiostimulant and psychostimulant properties of these benzofurans, similar to MDMA and methamphetamine. Unlike the relatively constant NET inhibition, the potencies of the benzofurans to inhibit the DAT and SERT notably varied, resulting in DAT : SERT inhibition ratios that ranged from 0.01 to 0.65. Specifically, the dihydrobenzofurans 5-APDB and 5-MAPDB exhibited the highest preference for the SERT versus DAT (more selective than MDMA), followed by 5-APB, 6-APDB and 5-EAPB, which exhibited a DAT : SERT inhibition ratio similar to MDMA. With DAT : SERT ratios of 0.46 and 0.65, 4-APB and 7-APB were the benzofurans with the most dopaminergic profiles and were relatively more dopaminergic than MDMA. Stimulants like methamphetamine exhibit a DAT : SERT ratio >10, whereas MDMA and other entactogens exhibit a DAT : SERT ratio of 0.01–1 (Simmler *et al.*, 2013; 2014a,b; Liechti, 2014b). Accordingly, based on their DAT : SERT inhibition ratios, all of the benzofurans can be expected to produce MDMA-like entactogenic subjective effects in humans. In contrast to the benzofurans, the benzodifuran 2C-B-FLY blocked monoamine transporters only at very high concentrations but had high affinity for 5-HT receptors. Thus, monoamine transporter inhibition is unlikely to contribute to the mechanism of action of 2C-B-FLY as is also the case for the structurally similar substance 2C-B and related compounds of the 2C phenethylamine series containing methoxy groups at positions 2 and 5 of the benzene ring (Acuna-Castillo *et al.*, 2002; Hill and Thomas, 2011; Eshleman *et al.*, 2014).

Only a few other studies have determined the monoamine transporter inhibition profiles of some of the benzofurans. Consistent with our findings, 5-APDB and 6-APDB inhibited the SERT more potently than the DAT in rat synaptosomes (Monte *et al.*, 1993). The oxygen in the para-position in the 5-APDB and 5-APB structures enhanced the 5-hydroxytryptaminergic versus dopaminergic properties compared with 6-APDB and 6-APB, respectively, as shown in the present study and previously for 5-APDB versus 6-APDB in rat synaptosomes (Monte *et al.*, 1993). Drug discrimination studies in rats showed that 5-APDB and 6-APDB substituted for MDMA-like 5-hydroxytryptaminergic drugs but not the more dopaminergic stimulant amphetamine (Monte *et al.*, 1993). These behavioural findings support our hypothesis that 5-APDB and 6-APDB produce subjective effects that are similar to MDMA, and entactogenic effects have been reported by users (Bluelight, 2013a,b; Drugs-Forum, 2013). The monoamine transporter inhibition profiles for 5-APB and 6-APB were determined in one previous study (Iversen *et al.*, 2013). In contrast to our results, this study showed that 5-APB and 6-APB inhibited the DAT more potently than the SERT (Iversen *et al.*, 2013). However, MDMA did not show the 5-hydroxytryptaminergic preference that is typically reported by others (Rothman *et al.*, 2001; Han and Gu, 2006; Hysek *et al.*, 2012c; Simmler *et al.*, 2013). Consistent with the present results, the inhibition profiles for 5-APB and 6-APB were similar to MDMA and unlike methamphetamine (Iversen *et al.*, 2013). The reinforcing effects of benzofurans have not yet been studied in drug self-administration studies. There is a decrease in reinforcing potency and efficacy among monoamine-releasing agents when 5-HT releasing potency is increased relative to dopamine (Wee *et al.*, 2005). The relatively high 5-hydroxytryptaminergic properties of the benzofurans *in vitro* would indicate lower addictive properties (Wee *et al.*, 2005; Liechti, 2014b), more similar to MDMA, which is

Table 2

Monoamine transporter and receptor-binding affinities

	NET	DAT	SERT	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	TA_{1rat}	TA_{1mouse}
Benzofurans											
5-APB	3.1 ± 0.2	2.6 ± 0.3	3.2 ± 0.4	3.5 ± 0.5	2.9 ± 0.1	>14	>10	>17	8.4 ± 0.8	0.04 ± 0.01	0.11 ± 0.01
5-APDB	28 ± 5	>30	4.0 ± 0.3	11 ± 3	4.2 ± 0.5	>14	>10	>17	21 ± 2	0.49 ± 0.05	0.77 ± 0.06
6-APB	1.8 ± 0.4	0.60 ± 0.05	12 ± 1	7.3 ± 3.4	0.38 ± 0.02	>14	>10	>17	15 ± 2	0.05 ± 0.02	0.06 ± 0.02
6-APDB	18 ± 1	>30	23 ± 1	>15	0.65 ± 0.07	>14	>10	>17	>25	1.0 ± 0.04	0.21 ± 0.04
5-MAPDB	26 ± 5	>30	6.3 ± 0.6	4.9 ± 1.5	6.4 ± 1.8	23 ± 3	>10	>17	4.9 ± 0.1	0.67 ± 0.09	3.5 ± 0.1
4-APB	3.9 ± 0.5	7.4 ± 0.6	7.7 ± 0.5	12 ± 3	0.87 ± 0.22	>14	>10	>17	16 ± 1	0.11 ± 0.02	2.08 ± 0.14
7-APB	5.3 ± 0.1	14 ± 2	14 ± 1	9.6 ± 2.4	0.14 ± 0.02	>14	8.2 ± 3.2	>17	25 ± 5	0.07 ± 0.01	0.13 ± 0.02
5-EAPB	1.0 ± 0.3	0.34 ± 0.02	0.52 ± 0.03	3.3 ± 0.5	2.7 ± 0.7	16 ± 3	>10	>17	2.4 ± 0.4	0.81 ± 0.08	>15
Benzodifuran											
2C-B-FLY	17 ± 4	>26	10 ± 3	11 ± 1	0.78 ± 0.3	1.4 ± 0.2	1.9 ± 0.3	6.8 ± 1.2	3.4 ± 0.5	0.03 ± 0.01	0.71 ± 0.23
Related amphetamines											
MDMA[a]	27 ± 9	8.4 ± 3.3	13 ± 2	>5	15 ± 10	>12	>20	>17	>13	0.37 ± 0.12	2.4 ± 1.1
MDA	13 ± 3.7	>26	5.6 ± 1.5	>5	1.1 ± 0.1	>12	>20	>17	>13	0.25 ± 0.04	0.16 ± 0.01
β-Keto-MDA	>30	11 ± 2	>30	>5	15 ± 2	>12	>20	>17	>13	4.8 ± 0.9	6.5 ± 2.8
Methamphetamine[a]	3.0 ± 2.2	1.8 ± 0.7	25 ± 10	>5	6.1 ± 1.6	>12	>20	>17	>13	0.35 ± 0.12	0.55 ± 0.24

Values are K_i given as μM (mean ± SD).
[a]Values are from Simmler *et al.*, 2014a.

Table 3

5-HT receptor interactions

	5-HT$_{1A}$	5-HT$_{2A}$			5-HT$_{2B}$		5-HT$_{2C}$
	Receptor-binding K_i (μM)	Receptor-binding K_i (μM)	Activation potency EC_{50} (μM)	Activation efficacy % maximum	Activation potency EC_{50} (μM)	Activation efficacy % maximum	Receptor-binding K_i (μM)
Benzofurans							
5-APB	3.3 ± 0.2	0.84 ± 0.27	6.3 ± 2.1	54 ± 35	0.28 ± 0.12	61 ± 17	0.88 ± 0.33
5-APDB	20 ± 4	3.4 ± 1.0	11 ± 2	24 ± 17	1.2 ± 0.6	50 ± 21	0.06 ± 0.02
6-APB	1.5 ± 0.2	0.97 ± 0.23	5.9 ± 1.8	43 ± 23	0.14 ± 0.06	70 ± 9	0.27 ± 0.05
6-APDB	9.2 ± 1.5	2.0 ± 1.0	5.9 ± 1.1	62 ± 36	0.12 ± 0.03	66 ± 17	0.06 ± 0.02
5-MAPDB	26 ± 6	4.8 ± 2.1	>20	0	>20	0	0.10 ± 0.02
4-APB	1.2 ± 0.1	0.96 ± 0.17	13 ± 2	30 ± 9	1.0 ± 0.5	38 ± 16	0.06 ± 0.02
7-APB	0.28 ± 0.05	0.91 ± 0.20	5.7 ± 2.0	43 ± 21	0.28 ± 0.52	52 ± 17	3.3 ± 0.3
5-EAPB	3.1 ± 0.6	2.7 ± 1.5	7.6 ± 3.2	29 ± 7	>20	0	4.6 ± 1.3
Benzodifuran							
2C-B-FLY	0.35 ± 0.04	0.011 ± 0.002	0.0015 ± 0.0002	82 ± 12	0.040 ± 0.036	56 ± 3	0.012 ± 0.004
Related amphetamines							
MDMA	12 ± 0.8[a]	6.3 ± 2.4	6.1 ± 0.3	55 ± 9	>20	0	>13[a]
MDA	4.9 ± 0.9	3.3 ± 0.8	0.63 ± 0.24	77 ± 16	0.85 ± 0.11	52 ± 12	3.0 ± 0.9
β-Keto-MDA	>17	>13	>20	0	>20	0	>13
Methamphetamine	8.1 ± 0.7[a]	>13	>20	0	>20	0	>13[a]

Values are K_i given as μM (mean ± SD).
[a]Values are from Simmler *et al.*, 2014a and were included for comparison.

not a strong reinforcer in self-administration studies (Lamb and Griffiths, 1987; Cole and Sumnall, 2003) than to methamphetamine.

All of the benzofurans also released 5-HT, NA and/or dopamine through their respective transporters, similar to their amphetamine analogues and other amphetamine derivatives (Simmler *et al.*, 2013; 2014a,b). Dopamine release has also been previously documented for 5-APB in voltammetric studies of rat brain slices (Dawson *et al.*, 2014). In contrast to the benzofurans, 2C-B-FLY did not release monoamines. Our release assay was designed to qualitatively assess monoamine release because we used only one high concentration of the substances to induce transporter-mediated monoamine efflux. Additional studies that include the assessment of transporter-mediated ionic currents and *in vivo* microdialysis could be useful to further characterize and quantify monoamine release and its contribution to the mechanism of action of the benzofurans.

Receptor-binding profiles

The present study found several important high-potency interactions between the benzofurans and various monoamine receptors. 6-APB, 6-APDB, 4-APB, 7-APB and 2C-B-FLY all bound to α_{2A}-adrenoceptors, which are known to modulate NA release and sympathomimetic activity (Hysek *et al.*, 2012a). As expected (Monte *et al.*, 1996), 2C-B-FLY potently interacted with 5-HT_2 receptors. Specifically, 2C-B-FLY potently bound to the human 5-HT_{2A} receptor (K_i = 0.01 μM), consistent with the previously documented nanomolar affinity for rat cortical 5-HT_{2A} receptors (Monte *et al.*, 1996). Even higher potency binding to 5-HT_{2A} receptors has been shown for the benzodifuran bromo-dragonFLY in rat (Monte *et al.*, 1996; Chambers *et al.*, 2001) and human (Monte *et al.*, 1996) 5-HT_{2A} receptors. In the present study, 2C-B-FLY was also a very potent functional 5-HT_{2A} receptor agonist. 2C-B-FLY resembles the structures of the 2C series phenethylamines, which are also potent 5-HT_{2A} receptor agonists (Nelson *et al.*, 1999; Acuna-Castillo *et al.*, 2002; Hansen *et al.*, 2014).

Consistent with the predicted lysergic acid diethylamide (LSD)-like properties of substances with high 5-HT_{2A} receptor affinity, both 2C-B-FLY and bromo-dragonFLY completely substituted for LSD in drug discrimination studies (Monte *et al.*, 1996). The affinity of 2C-B-FLY for the 5-HT_{1A} receptor was relatively low, which has also been shown for rat 5-HT_{1A} receptors (Monte *et al.*, 1996). The 5-HT_{2A} receptor is thought to mediate the alterations in perception induced by hallucinogens (Vollenweider *et al.*, 1998; Nelson *et al.*, 1999; Nichols, 2004) and therefore is likely to be the key target in the mechanism of action of benzodifuran hallucinogens. Interestingly, some of the benzofurans also exhibited micromolar affinity for the 5-HT_{2A} receptor and were low-potency 5-HT_{2A} receptor partial agonists similar to MDMA and MDA, but in contrast to methamphetamine. Binding to 5-HT_{2A} receptors at micromolar concentrations has also been previously shown for 5-APB and 6-APB (Iversen *et al.*, 2013). 5-APB also constricts the rat aorta via an agonist action on 5-HT_{2A} receptors (Dawson *et al.*, 2014). Thus, some benzofurans could have hallucinogenic properties because of 5-HT_{2A} receptor stimulation, in addition to their MDMA-like entactogenic subjective effects. Psychosis and hallucinations have been reported after the use of 6-APB (Chan *et al.*, 2013; Greene, 2013). However, in drug discrimination studies, 5-APDB and 6-APDB did not substitute for LSD in rats (Monte *et al.*, 1993), consistent with their lower binding affinity compared with 5-APB and 6-APB. In terms of clinical toxicity, the 5-HT_{2A} receptor agonist and possible α_1-adrenoceptor agonist action could enhance the risk for vasoconstriction, hyperthermia and hypertension. Both α_1 and 5-HT_{2A} receptors are implicated in substance-induced vasoconstriction (Blessing *et al.*, 2003; Docherty and Green, 2010; Dawson *et al.*, 2014) and associated hypertension (Hysek *et al.*, 2013) and hyperthermia (Liechti *et al.*, 2000; Hysek *et al.*, 2012b; Liechti, 2014a) in humans. In fact, hypertension, hyperpyrexia and cases of severe limb ischaemia have been reported after the use of bromo-dragonFLY (Thorlacius *et al.*, 2008; Wood *et al.*, 2009; Nielsen *et al.*, 2010), a benzodifuran structurally similar to 2C-B-FLY. Direct agonist actions at the 5-HT_{2A} receptor compared with an indirect action via 5-HT release can also be expected to result in longer lasting effects as described for 5-hydroxytryptaminergic hallucinogens (Schmid *et al.*, 2014) and compared with MDMA (Hysek *et al.*, 2012c).

In humans, MDMA is mainly inactivated by *O*-demethylation but also *N*-demethylated to the minor but active metabolite MDA (Hysek *et al.*, 2013). Similarly, 5-MAPDB and 5-EAPB are *N*-dealkylated (Welter *et al.*, 2015) to 5-APDB and 5-APB respectively. As shown in the present study, the *N*-dealkylated substances MDA, 5-APDB and 5-APB activate 5-HT_{2A} and 5-HT_{2B} receptors more potently and also more potently bind to 5-HT_{2C} receptors than their parent compounds MDMA, 5-MAPDB and 5-EAPB respectively. Thus, the formation of active metabolites probably adds enhanced 5-HT_{2A} and 5-HT_{2B} receptor-associated toxicity in these cases.

2C-B-FLY and several of the benzofurans acted as partial agonists at the 5-HT_{2B} receptor as previously shown for 5-APB (Iversen *et al.*, 2013; Dawson *et al.*, 2014) and 6-APB (Iversen *et al.*, 2013). In contrast, no such 5-HT_{2B} receptor agonist properties were observed for the classic amphetamines MDMA and methamphetamine in the present study. 5-HT_{2B} receptors have been implicated in substance-induced heart valve fibrosis (Setola *et al.*, 2003; Bhattacharyya *et al.*, 2009). 5-HT_{2B} receptor activation by 2C-B-FLY, 5-APB, 6-APB, 6-APDB and 7-APB occurred at submicromolar concentrations that are likely to be present when these drugs are used by drug users to induce subjective effects.

All of the benzofurans bound to TA_1 receptors, many at even higher potency than MDMA or methamphetamine. MDMA and methamphetamine inhibit their own neurochemical and locomotor stimulant effects via TA_1 receptor activation (Di Cara *et al.*, 2011). Similar TA_1 receptor-mediated 'auto-inhibition' may, therefore, modulate the effects of benzofurans. In contrast, for cathinones (e.g. β-keto-amphetamines), more stimulant-like and addictive properties would be expected based on their lower affinity for TA_1 receptors compared with their amphetamine analogues (Simmler *et al.*, 2013; 2014a).

In terms of structure–activity relationships (Table 4), 3,4-substitution on the benzene ring (methylenedioxy group in MDMA and MDA, furans or dihydrofurans) strongly reduced the DAT/SERT inhibition ratio confirming previous studies (Nichols, 1994; Han and Gu, 2006; Iversen *et al.*, 2013;

Table 4

Structure–activity relationships

Structure	Present in	Not present in	Pharmacolocial (clinical) activity
3,4-Substitution on benzene ring (methylenedioxy or furan)	MDMA, 5-MAPDB, 5-EAPB MDA, 5-APB, 6-APB, 5-APDB, 6-APDB, 7-APB, β-keto-MDA	Methamphetamine [a]Amphetamine	Reduced DAT/SERT inhibition ratio, [a]increased potency to release 5-HT, [a]reduced potency to release dopamine (more entactogenic, less stimulant)
Oxygen in para-(4)-position	5-APB, 5-APDB, 5-MAPDB	7-APB, 4-APB, 6-APB, 6-APDB	Reduced DAT/SERT inhibition ratio (more serotonergic)
Dihydrobenzofuran	5-APDB, 6-APDB, 5-MAPDB	5-APB, 6-APB, 4-APB, 7-APB	Reduced DAT/SERT inhibition ratio (more 5-hydroxytryptaminergic)
N-Alkyl group	MDMA, 5-EAPB, 5-MAPDB	MDA, 5-APB, 5-APDB	Reduced 5-$HT_{2A/B}$ receptor activation and 5-HT_{2C} receptor-binding potency (less hallucinogenic)
2,5-Oxy-substitution on benzene ring	2C-B-FLY	All other compounds	Strongly increased 5-$HT_{2A/B}$ receptor activation, strongly increased 5-HT_{2C} receptor-binding potency (more hallucinogenic)
β-Keto group	β-Keto MDA	MDA	[a,b]Increased DAT/SERT ratio (more dopaminergic)

[a]Simmler *et al.*, 2013.
[b]Simmler *et al.*, 2014a.

Simmler *et al.*, 2013). Additionally, the DAT/SERT inhibition ratio depended on the position of the oxygen on the benzene ring and was lowest for compounds with the oxygen in the para-(4)-position and highest for those with the oxygen in the ortho-(2)-position. This finding was consistent with the high 5-hydroxytryptaminergic activity of other para-substituted amphetamines (Nichols, 1994; Rickli *et al.*, 2015). The dihydrobenzofurans (5-APDB, 6-APDB and 5-MAPDB) exhibited reduced monoamine transporter inhibition potency in particular at the DAT resulting in relatively more 5-hydroxytryptaminergic properties compared with their furan analogues. *N*-alkylation (MDMA, 5-MAPDB, 5-EAPB, methamphetamine) moderately reduced activity at 5-$HT_{2A/B}$ receptors and binding at 5-HT_{2C} receptors. This has previously been shown for other phenethylamines for simple *N*-alkylation (e.g. methyl, ethyl) (Nelson *et al.*, 1999). *N*-alkylation had no relevant effect on the interactions with the monoamine transporter as previously noted for related amphetamines (Nichols, 1994). 2,5-Substitution on the benzene ring strongly increased activity at the 5-HT_2 receptors and reduced interactions with the monoamine transporters as seen in 2C-B-FLY in the present study and many other 2,5-substituted phenethylamines (2C series) (Hill and Thomas, 2011; Eshleman *et al.*, 2014). Transporter inhibition potency was also moderately reduced when the oxygen was in the ortho-(2)-position (similar to the 2 series) at the benzene ring as in 7-APB compared with 5-APB, 6-APB or 4-APB. β-Keto-substitution increased dopaminergic versus 5-hydroxytryptaminergic activity extending previous similar findings (Simmler *et al.*, 2013; 2014a).

Conclusions

Benzofurans are monoamine transporter blockers and monoamine releasers, similar to MDMA, but they also interact with 5-HT receptors. This mechanism of action predicts psychotropic and clinical toxicological effects that are similar to the entactogen MDMA but with additional hallucinogenic properties. The benzodifuran 2C-B-FLY is a potent hallucinogen, probably also associated with a risk for clinical complications related to vasoconstriction (e.g. ischaemia and hypertension). Although structure–activity relationships exist, the present study showed that structurally very similar compounds may exhibit distinct pharmacological profiles, illustrating the need for pharmacotoxicological profiling of each novel psychoactive substance.

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Author contributions

A. R. and M. E. L. designed the research study. A. R., S. K. and M. C. H. performed the research. A. R., M. C. H. and M. E. L. analysed the data. A. R. and M. E. L. wrote the paper.

Conflict of interest

None.

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Paper 6

Receptor interaction profiles of novel N-2-methoxybenzyl (NBOMe) derivatives of 2,5-dimethoxy-substituted phenethylamines (2C drugs).

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Receptor interaction profiles of novel *N*-2-methoxybenzyl (NBOMe) derivatives of 2,5-dimethoxy-substituted phenethylamines (2C drugs)

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ABSTRACT

Background: *N*-2-methoxybenzyl-phenethylamines (NBOMe drugs) are newly used psychoactive substances with poorly defined pharmacological properties. The aim of the present study was to characterize the receptor binding profiles of a series of NBOMe drugs compared with their 2,5-dimethoxy-phenethylamine analogs (2C drugs) and lysergic acid diethylamide (LSD) *in vitro*.
Methods: We investigated the binding affinities of 2C drugs (2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, and mescaline), their NBOMe analogs, and LSD at monoamine receptors and determined functional 5-hydroxytryptamine-2A (5-HT_{2A}) and 5-HT_{2B} receptor activation. Binding at and the inhibition of monoamine uptake transporters were also determined. Human cells that were transfected with the respective human receptors or transporters were used (with the exception of trace amine-associated receptor-1 [$TAAR_1$], in which rat/mouse receptors were used).
Results: All of the compounds potently interacted with serotonergic 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors and rat $TAAR_1$ (most K_i and EC_{50}: <1 μM). The *N*-2-methoxybenzyl substitution of 2C drugs increased the binding affinity at serotonergic 5-HT_{2A}, 5-HT_{2C}, adrenergic α_1, dopaminergic $D_{1\text{-}3}$, and histaminergic H_1 receptors and monoamine transporters but reduced binding to 5-HT_{1A} receptors and $TAAR_1$. As a result, NBOMe drugs were very potent 5-HT_{2A} receptor agonists (EC_{50}: 0.04–0.5 μM) with high $5\text{-HT}_{2A}/5\text{-HT}_{1A}$ selectivity and affinity for adrenergic α_1 receptors (K_i: 0.3–0.9 μM) and $TAAR_1$ (K_i: 0.06–2.2 μM), similar to LSD, but not dopaminergic D_{1-3} receptors (most K_i: > 1 μM), unlike LSD.
Conclusion: The binding profile of NBOMe drugs predicts strong hallucinogenic effects, similar to LSD, but possibly more stimulant properties because of α_1 receptor interactions.

Abbreviations: 25B-NBOMe, 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25C-NBOMe, 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25D-NBOMe, 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25E-NBOMe, 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25H-NBOMe, 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25I-NBOMe, 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25N-NBOMe, 2-(4-nitro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25P-NBOMe, 2-(4-propyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T2-NBOMe, 2-(2,5-dimethoxy-4-ethylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T4-NBOMe, 2-(2,5-dimethoxy-4-isopropylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T7-NBOMe, 2-(2,5-dimethoxy-4-*n*-propylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 2C-C, 2-(4-chloro-2,5-dimethoxy)ethanamine; 2C-D, 2-(2,5-dimethoxy-4-methyl) ethanamine; 2C-E, 1-(2,5-dimethoxy-4-ethylphenyl)-2-aminoethane; 2C-H, 2,5-dimethoxyphenethylamine; 2C-I, 4-iodo-2,5-dimethoxyphenethylamine; 2C-N, 2-(2,5-dimethoxy-4-nitro)ethanamine; 2C-P, 2-(2,5-dimethoxy-4-propylphenyl)ethanamine; 25CN-NBOH, 2-([2-(4-cyano-2,5-dimethoxyphenyl)ethylamino]-methyl)phenol; 2C-T-2, 2-[2,5-dimethoxy-4-(ethylthio)phenyl]ethanamine; 2C-T-4, 2,5-dimethoxy-4-isopropylthiophenethylamine; 2C-T-7, 2-[2,5-dimethoxy-4-(propylthio)phenyl]ethanamine; 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DAT, dopamine transporter; mescaline, 2-(3,4,5-trimethoxyphenyl)ethanamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; NBOMe, *N*-(2-methoxy)benzyl; NE, norepinephrine; NET, norepinephrine transporter; SERT, serotonin transporter; TAAR, trace amine-associated receptor; LSD, lysergic acid diethylamide.

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1. Introduction

New psychoactive substances are constantly emerging on the illicit drug market and typically sold via the Internet. Of particular interest are *N*-2-methoxybenzyl-phenethylamines (NBOMe drugs), which are novel and reportedly very potent hallucinogens that have been increasingly used recreationally (Forrester, 2014; Hill et al., 2013; Ninnemann and Stuart, 2013; Rose et al., 2013; Walterscheid et al., 2014; Wood et al., 2015; Zuba, 2012), with additional potential use as radiotracers (Ettrup et al., 2011, 2010). Recreationally used NBOMe drugs include 25I-NBOMe, 25C-NBOMe, 25B-NBOMe, and 25D-NBOMe (Armenian and Gerona, 2014; Poklis et al., 2014; Rose et al., 2013), which are derivatives of 2,5-dimethoxy-4-substituted phenethylamines (2C drugs; Dean et al., 2013; Hill and Thomas, 2011; Shulgin and Shulgin, 1991) (see Fig. 1). *N*-2-methoxybenzyl substitution enhances the potency of 2C drugs at serotonergic 5-hydroxytryptamine-2A (5-HT_{2A}) receptors, resulting in exceptionally potent 5-HT_{2A} receptor agonists (Braden et al., 2006; Heim, 2004; Nichols et al., 2015) with strong hallucinogenic properties in animals and humans (Halberstadt and Geyer, 2014; Srisuma et al., 2015). Pharmacological interactions between NBOMe drugs and 5-HT_2 receptors have been well characterized for some compounds of this novel drug family (Blaazer et al., 2008; Braden et al., 2006; Ettrup et al., 2011, 2010; Hansen et al., 2014; Nichols et al., 2008). However, systematic characterizations of the effects of a larger series of NBOMe drugs at a wider range of relevant human receptors and comparisons with their 2C parent drugs are lacking. Importantly, NBOMe drugs have been reported to produce psycho- and cardiovascular stimulant effects, in addition to hallucinations. Specifically, sympathomimetic toxicity, including tachycardia, hypertension, mydriasis, agitation, and hyperthermia, is commonly reported in cases of acute NBOMe drug intoxication (Hill et al., 2013; Rose et al., 2013; Srisuma et al., 2015; Stellpflug et al., 2014; Wood et al., 2015). Pharmacologically, compounds of the 2C series, including 2C-C, 2C-E, and 2C-I, inhibit the norepinephrine (NE) and serotonin transporters (NET and SERT, respectively), similar to amphetamines, although with only very low potency (Eshleman et al., 2014; Nagai et al., 2007). These findings raise the question of whether NBOMe drugs may have similar but more potent stimulant-type pharmacological properties, including inhibition of the NET, dopamine (DA) transporter (DAT), and SERT, or interactions with adrenergic α_1 receptors that lead to vasoconstriction.

We assessed the *in vitro* pharmacology of a series of NBOMe drugs compared with their 2C parent drugs. We characterized the binding affinity profiles at monoamine receptors and DAT, NET, and SERT inhibition potencies. We also determined the functional 5-HT_{2A} receptor activation potencies because 5-HT_{2A} receptors mediate hallucinogenic effects (Nichols, 2004). The prototypical serotonergic hallucinogen lysergic acid diethylamide (LSD) was included as a comparator drug (Nichols, 2004; Passie et al., 2008).

2. Methods

2.1. Drugs

2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, mescaline, 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe, 25T7-NBOMe, and mescaline-NBOMe were synthesized by Lipomed (Arlesheim, Switzerland) for this study at no cost. All of the compounds were used as hydrochloride salts. Purity was >98% for all of the substances. [^{3}H]NE and [^{3}H]DA were obtained from Perkin–Elmer (Schwerzenbach, Switzerland), and [^{3}H]5-HT was obtained from Anawa (Zürich, Switzerland).

2.2. Radioligand receptor and transporter binding assays

The radioligand binding assays were performed as described previously (Hysek et al., 2012; Simmler et al., 2013). Briefly, membrane preparations of human embryonic kidney (HEK) 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective transporters (Tatsumi et al., 1997) or receptors (human genes, with the exception of rat and mouse genes for trace amine-association receptor 1 [$TAAR_1$]; (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 that was 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*-propylamine)tetralin and indatraline (5-HT_{1A} receptor), [^{3}H]ketanserin and spiperone (5-HT_{2A} receptor), [^{3}H]mesulgerine and mianserin (5-HT_{2C} receptor), [^{3}H]prazosin and risperidone (adrenergic α_1 receptor), [^{3}H]rauwolscine and phentolamine (adrenergic α_2 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 non-linear 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.

2.3. Activity at serotonin 5-HT_{2A} receptor

Human 5-HT_{2A} receptor-expressing NIH-3T3 cells were incubated in HEPES- Hank's Balanced Salt Solution (HBSS) buffer (70′000 cells/100 μl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. To each well 100 μl of Dye solution (FLIPR calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added and plates were incubated for 1 h at 37 °C. The plates were then placed in a fluorescence imaging plate reader (FLIPR), and 25 μl of the test substances diluted in HEPES-HBSS buffer containing 250 mM probenicid were added online. 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%.

2.4. Activity at serotonin 5-HT_{2B} receptor

Human 5-HT_{2B} receptor-expressing HEK293 cells were incubated in growth medium (DMEM high glucose [Invitrogen, Zug, Switzerland], 10 ml/l PenStrep [Gibco, Life Technologies, Zug, Switzerland]), 10% FCS non dialyzed heat inactivated and 250 mg/l geneticin) at a density of 50′000 cells/well at 37 °C in 96-well poly-D-lysine-coated plates over-night. On the next day the growth medium was removed by snap inversion, and 100 μl of Fluo-4 solution (calcium indicator; Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C. The Fluo-4 solution was removed by snap inversion, and 100 μl of Fluo-4 solution was added a second time. The cells were then incubated for another 45 min at 31 °C. 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

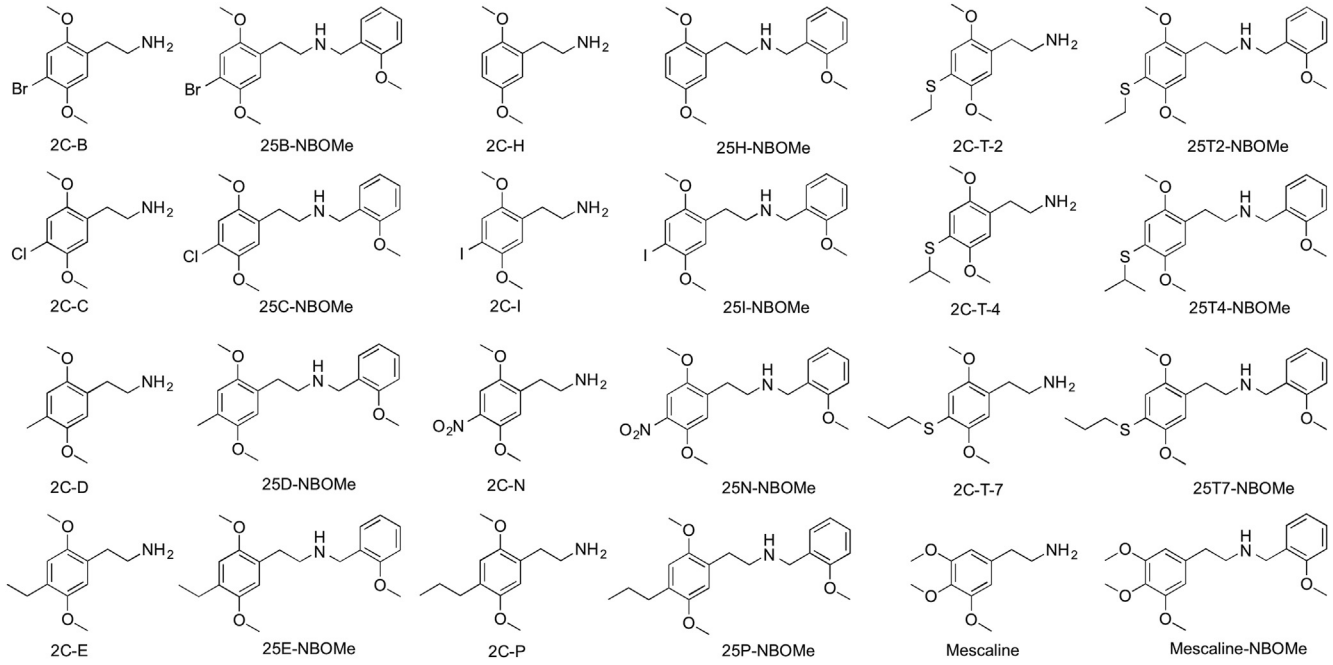

Fig. 1. Chemical structures of 2,5-dimethoxyphenethylamines (2C drugs) and their *N*-2-methoxybenzyl-substituted analogs (NBOMe drugs).

buffer was added online. 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%.

2.5. Monoamine uptake transporter inhibition

Inhibition of the human NET, DAT, and SERT was assessed in HEK 293 cells that were stably transfected with transporters as specified previously (Hysek et al., 2012). Briefly, the cells were suspended in uptake buffer and incubated for 10 min with different concentrations of the test substances. The corresponding radiolabeled [^{3}H] monoamine (5 nM final concentration) was then added at room temperature. After 10 min, uptake was stopped by separating the cells from the buffer using centrifugation through silicone oil (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 β-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%). The data were fitted by non-linear regression to variable slope sigmoidal dose–response curves (bottom = 0%), and IC_{50} values were calculated using Prism software (GraphPad, San Diego, CA, USA).

2.6. Cytotoxicity

To confirm cell integrity during the pharmacological assays, cytotoxicity was assessed using the ToxiLight bioassay (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The assay quantitatively measures the release of adenylate kinase from damaged cells, providing a highly sensitive method of measuring cytolysis (Crouch et al., 1993). Cells that were grown in 96-well plates were exposed to the compounds at a high concentration of 100 μM. All of the test conditions contained 0.1% (v:v) dimethylsulfoxide, which is non-toxic at this concentration and was also used as a negative control. Triton X-100 (0.1%, Sigma–Aldrich, Buchs, Switzerland) lyses cells and was used as a positive control. After 4 h incubation at 37 °C, 10 μl of the supernatant per well was removed and combined with 50 μl of ToxiLight reagent, and luminescence was recorded using a Tecan Infinite 200 Pro plate reader (Tecan, Männedorf, Switzerland).

3. Results

3.1. Interactions with serotonin receptors

Table 1 shows binding to serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, activation potency and efficacy at 5-HT_{2A} and 5-HT_{2B} receptors, and 5-HT receptor binding ratios. All of the compounds exhibited high binding affinity for 5-HT_{2A} and 5-HT_{2C} receptors (K_i < 1 μM, with the exception of 2C-H and mescaline). *N*-2-methoxybenzyl substitution further increased the average binding affinity for both 5-HT_{2A} and 5-HT_{2C} receptors 26- and 14-fold (range: 6–100 and 8–32, respectively), leading to compounds with up to 8.4-fold higher affinity for these receptors compared with LSD. Moderate 5-HT_{2A} over 5-HT_{2C} receptor binding preference was observed, with 5-HT_{2A}/5-HT_{2C} receptor binding ratios of 3–16 for the 2C drugs and slightly more selective ratios of 5–26 for the NBOMe drugs. All of the compounds also potently activated 5-HT_{2A} receptors and typically more potently than LSD (EC_{50} < 1 μM, with the exception of 2C-H, mescaline, and mescaline-NBOMe). However, in contrast to the robust effect on binding to 5-HT_{2A} receptors, *N*-2-methoxybenzyl substitution did not consistently change the activation potency at 5-HT_{2A} receptors and even reduced the activation efficacy, with the exception of 2C-H. All of the compounds potently activated the 5-HT_{2B} receptor (EC_{50} < 1 μM, with the exception of 2C-H, mescaline, mescaline-NBOMe, and LSD). *N*-2-methoxybenzyl substitution increased 5-HT_{2B} receptor activation 5-fold (range: 0.8–18) but reduced activation efficacy. All of the 2C drugs potently bound to 5-HT_{1A}

Table 1
Serotonin receptor interactions.

	5-HT$_{1A}$	5-HT$_{2A}$			5-HT$_{2B}$		5-HT$_{2C}$	Selectivity (binding ratios)	
	Receptor binding K_i ± SD [μM]	Receptor binding K_i ± SD [μM]	Activation potency EC_{50} ± SD [μM]	Activation efficacy % maximum ± SD	Activation potency EC_{50} ± SD [μM]	Activation efficacy % maximum ± SD	Receptor binding K_i ± SD [μM]	5-HT$_{2A}$/ 5-HT$_{1A}$	5-HT$_{2A}$/ 5-HT$_{2C}$
2Cs									
2C-B	0.24 ± 0.04	0.0086 ± 0.003	0.08 ± 0.02	45 ± 7	0.13 ± 0.06	89 ± 13	0.047 ± 0.009	28	4.7
2C-C	0.19 ± 0.01	0.0130 ± 0.005	0.20 ± 0.06	49 ± 10	0.28 ± 0.11	81 ± 14	0.090 ± 0.026	15	6.9
2C-D	0.44 ± 0.01	0.0324 ± 0.005	0.35 ± 0.18	41 ± 3	0.23 ± 0.07	77 ± 17	0.15 ± 0.03	14	4.6
2C-E	0.36 ± 0.04	0.0105 ± 0.001	0.11 ± 0.03	40 ± 2	0.19 ± 0.04	66 ± 7	0.10 ± 0.02	34	10
2C-H	0.07 ± 0.02	1.6 ± 0.3	9.4 ± 0.5	28 ± 5	6.2 ± 2.8	46 ± 18	4.1 ± 0.9	0.04	2.6
2C-I	0.18 ± 0.01	0.0035 ± 0.001	0.06 ± 0.03	45 ± 8	0.15 ± 0.10	70 ± 18	0.040 ± 0.009	51	11
2C-N	2.2 ± 0.1	0.0235 ± 0.011	0.17 ± 0.04	48 ± 10	0.73 ± 0.09	74 ± 20	0.37 ± 0.02	94	16
2C-P	0.11 ± 0.04	0.0081 ± 0.001	0.09 ± 0.06	63 ± 5	0.13 ± 0.01	72 ± 18	0.040 ± 0.005	14	4.9
2C-T-2	0.37 ± 0.04	0.0090 ± 0.002	0.08 ± 0.03	67 ± 16	0.13 ± 0.09	75 ± 14	0.069 ± 0.018	41	7.7
2C-T-4	0.47 ± 0.13	0.0279 ± 0.012	0.22 ± 0.13	87 ± 7	0.16 ± 0.06	68 ± 10	0.18 ± 0.07	17	6.5
2C-T-7	0.52 ± 0.05	0.0065 ± 0.002	0.13 ± 0.05	76 ± 10	0.35 ± 0.25	45 ± 10	0.039 ± 0.013	80	6.0
Mescaline	4.6 ± 0.4	6.3 ± 1.8	10 ± 1.8	56 ± 15	>20	NA	17 ± 2.0	0.73	2.7
N-benzylphenylethylamines (NBOMes)									
25B-NBOMe	3.6 ± 0.3	0.0005 ± 0.0000	0.04 ± 0.01	28 ± 7	0.01 ± 0.01	19 ± 5	0.0062 ± 0.0022	7200	12
25C-NBOMe	5.0 ± 0.1	0.0007 ± 0.0002	0.15 ± 0.06	32 ± 2	0.10 ± 0.13	16 ± 5	0.0052 ± 0.0026	7143	7.4
25D-NBOMe	7.1 ± 0.5	0.0010 ± 0.0004	0.09 ± 0.03	27 ± 7	0.10 ± 0.07	22 ± 6	0.013 ± 0.004	7100	13
25E-NBOMe	3.5 ± 0.2	0.0006 ± 0.0001	0.16 ± 0.11	28 ± 15	0.06 ± 0.03	26 ± 10	0.0072 ± 0.0029	5833	12
25H-NBOMe	6.0 ± 0.7	0.0164 ± 0.0014	0.49 ± 0.07	38 ± 10	0.34 ± 0.14	11 ± 5	0.13 ± 0.02	366	7.9
25I-NBOMe	1.8 ± 0.3	0.0006 ± 0.0002	0.24 ± 0.12	27 ± 7	0.13 ± 0.08	32 ± 12	0.0046 ± 0.0020	3000	7.7
25N-NBOMe	4.2 ± 0.6	0.0008 ± 0.0002	0.07 ± 0.03	34 ± 3	0.07 ± 0.03	26 ± 14	0.021 ± 0.003	5250	26
25P-NBOMe	1.8 ± 0.1	0.0011 ± 0.0002	0.22 ± 0.11	42 ± 7	0.17 ± 0.13	23 ± 8	0.0060 ± 0.0015	1636	5.5
25T2-NBOMe	2.2 ± 0.2	0.0006 ± 0.0002	0.10 ± 0.03	38 ± 6	0.04 ± 0.04	31 ± 12	0.0065 ± 0.0006	3667	11
25T4-NBOMe	2.5 ± 0.3	0.0016 ± 0.0004	0.13 ± 0.05	46 ± 8	0.20 ± 0.10	27 ± 11	0.016 ± 0.005	1563	10
25T7-NBOMe	1.8 ± 0.2	0.0011 ± 0.0002	0.26 ± 0.16	41 ± 6	0.31 ± 0.23	14 ± 5	0.0064 ± 0.0013	1636	5.8
Mescaline-NBOMe	21 ± 5.7	0.14 ± 0.03	3.0 ± 0.6	33 ± 11	>20	NA	0.64 ± 0.04	147	4.5
LSD	0.0030 ± 0.0005	0.0042 ± 0.0013	0.26 ± 0.15	28 ± 10	12 ± 0.35	71 ± 31	0.015 ± 0.003	0.71	3.6

Values are K_i given as μM (mean ± SD); NA, not assessed.

receptors (K_i < 0.52 μM, with the exception of 2C-N and mescaline), although none exhibited the very high affinity of LSD. *N*-2-methoxybenzyl substitution decreased binding to 5-HT$_{1A}$ on average 17-fold (range: 2–86). The 2C drugs preferentially bound to 5-HT$_{2A}$ over 5-HT$_{1A}$ receptors with binding ratios of 14–94, with the exception of 2C-H and mescaline (Table 1). Receptor selectivity was markedly increased for 5-HT$_{2A}$ over 5-HT$_{1A}$ receptors for all of the compounds with *N*-2-methoxybenzyl substitution, with 5-HT$_{2A}$/5-HT$_{1A}$ ratios >100 for 25H-NBOMe and mescaline-NBOMe and >1000 for all of the other NBOMe drugs.

3.2. Binding to monoamine receptors and transporters

Table 2 shows the binding affinities for monoamine receptors and transporters. Compared with the 2C drugs, the NBOMe analogs exhibited higher binding affinities for all receptors and transporters, with the exception of TAAR$_1$. Specifically, all of the NBOMe drugs and LSD showed high-affinity binding to adrenergic α_{1A} receptors (K_i < 1 μM, with the exception of mescaline-NBOMe) and 19-fold (range: 11–38) higher binding affinity compared with the 2C drugs (not including mescaline). Most of the compounds also potently bound to α_{2A} receptors (K_i < 1 μM, with the exception of 2C-H, 2C-N, and mescaline). *N*-2-methoxybenzyl substitution did not appreciably alter α_{2A} receptor binding. LSD was the only substance that exhibited high-affinity binding to dopamine D_1-D_3 receptors. Most of the 2C and NBOMe drugs showed low-affinity binding to D_2 receptors, and NBOMe drugs also showed low-affinity binding to D_2 and D_3 receptors. *N*-2-methoxybenzyl substitution also increased histamine H_1 receptor binding 65-fold (range: 2–267) compared with the 2C analogs, resulting in high-affinity binding for several NBOMe drugs (Table 2). All of the 2C and NBOMe drugs showed high-affinity binding to TAAR$_{1rat}$ (K_i < 1 μM, with the exception of mescaline, 25-H-NBOMe, 25-N-NBOMe, and mescaline-NBOMe). *N*-2-methoxybenzyl substitution decreased binding to TAAR$_{1rat}$ 4-fold (range: 2–9). Binding affinity to monoamine transporters was low for 2C drugs (K_i > 10 μM). *N*-2-methoxybenzyl substitution increased binding to all monoamine transporters, resulting in low-affinity interactions for most of the NBOMe drugs (K_i < 1–10 μM, with the exception of mescaline-NBOMe). LSD did not interact with any of the monoamine transporters.

3.3. Monoamine uptake transporter inhibition

IC_{50} values for monoamine uptake inhibition are listed in Table 3. The 2C drugs did not inhibit or only very weakly inhibited (IC_{50} > 10 μM) monoamine uptake. *N*-2-methoxybenzyl substitution consistently enhanced monoamine uptake inhibition potency approximately two-to 15-fold for the NET, two-to five-fold for the DAT, and two-to 26-fold for the SERT. As a result, 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, and 25I-NBOMe blocked the NET and/or SERT at 5–10 μM concentrations. LSD did not inhibit any of the monoamine transporters.

3.4. Cytotoxicity

None of the compounds produced cytotoxicity after 4 h incubation at 37 °C, with the exception of 25T7-NBOMe. 25T7-NBOMe became toxic after 4 h incubation at 100 μM (but not 10 μM). Because the assays lasted less than 4 h, this toxicity did not affect the data.

Table 2
Monoamine transporter and receptor-binding affinities.

	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$	NET[a]	DAT[b]	SERT[c]
2C-series											
2C-B	8.2 ± 2.2	0.32 ± 0.01	12 ± 1.2	2.2 ± 0.3	10 ± 2.0	14 ± 0.5	0.09 ± 0.01	3.0 ± 0.3	31 ± 6.6	>30	9.7 ± 0.3
2C-C	13 ± 1.9	0.53 ± 0.06	13 ± 1.0	2.1 ± 0.4	17 ± 0.3	24 ± 0.9	0.11 ± 0.02	4.1 ± 0.3	>30	>30	24 ± 4.1
2C-D	12 ± 3.2	0.29 ± 0.03	24 ± 5.2	7.1 ± 1.7	>17	>25	0.15 ± 0.03	3.5 ± 0.1	>30	>30	31 ± 2.2
2C-E	7.4 ± 2.8	0.10 ± 0.02	15 ± 0.6	3.2 ± 1.0	19 ± 4.4	>25	0.07 ± 0.01	1.2 ± 0.1	33 ± 2.7	>30	29 ± 4.4
2C-H	7.9 ± 1.8	1.0 ± 0.05	>14	9.0 ± 1.5	>17	>25	0.90 ± 0.16	11 ± 2.2	>30	>30	>30
2C-I	5.1 ± 1.1	0.07 ± 0.01	13 ± 4.1	2.7 ± 0.58	5.0 ± 0.1	6.1 ± 0.5	0.12 ± 0.02	3.3 ± 0.1	15 ± 3.5	>30	4.9 ± 0.3
2C-N	>15	1.3 ± 0.2	19 ± 5.2	6.1 ± 2.7	20 ± 3.1	>25	0.34 ± 0.02	>20	>30	>30	32 ± 3.1
2C-P	3.5 ± 0.5	0.09 ± 0.01	8.4 ± 0.9	2.3 ± 0.7	5.2 ± 0.5	21 ± 3.2	0.02 ± 0.01	0.28 ± 0.03	18 ± 2.4	40 ± 4.0	19 ± 0.2
2C-T-2	17 ± 6.4	0.23 ± 0.01	15 ± 1.7	5.1 ± 1.0	11 ± 0.6	>25	0.04 ± 0.01	2.2 ± 0.6	>30	>30	13 ± 0.6
2C-T-4	11 ± 4.4	0.13 ± 0.04	20 ± 6.3	16 ± 2.1	19 ± 1.4	>25	0.05 ± 0.01	4.5 ± 0.9	17 ± 1.1	>30	>30
2C-T-7	13 ± 5.0	0.18 ± 0.001	15 ± 3.1	5.0 ± 0.8	7.5 ± 0.3	>25	0.03 ± 0.01	0.56 ± 0.1 2	27 ± 9.8	34 ± 6.2	12 ± 0.7
Mescaline	>15	1.4 ± 0.2	>14	>10	>17	>25	3.3 ± 0.5	11 ± 3.6	>30	>30	>30
N-benzylphenylethylamines (NBOMes)											
25B-NBOMe	0.43 ± 0.10	0.43 ± 0.03	9.3 ± 2.0	0.84 ± 0.27	2.7 ± 0.3	0.08 ± 0.02	0.28 ± 0.002	4.5 ± 1.7	1.1 ± 0.3	7.2 ± 0.5	0.84 ± 0.06
25C-NBOMe	0.81 ± 0.26	0.56 ± 0.08	12 ± 1.6	1.6 ± 0.4	3.5 ± 0.3	0.09 ± 0.01	0.52 ± 0.10	15 ± 1.9	1.6 ± 0.6	14 ± 3	1.5 ± 0.1
25D-NBOMe	0.70 ± 0.26	0.37 ± 0.05	8.7 ± 1.4	2.6 ± 0.4	6.4 ± 0.9	0.63 ± 0.06	0.81 ± 0.10	13 ± 4.4	2.2 ± 0.3	14 ± 2.4	1.4 ± 0.2
25E-NBOMe	0.53 ± 0.20	0.26 ± 0.07	4.9 ± 0.9	1.5 ± 0.2	3.2 ± 0.2	1.4 ± 0.2	0.26 ± 0.03	1.1 ± 0.3	3.0 ± 0.2	8.1 ± 0.6	1.7 ± 0.1
25H-NBOMe	0.55 ± 0.05	0.53 ± 0.04	14 ± 2.4	7.7 ± 1.7	20 ± 4.5	4.1 ± 0.4	1.4 ± 0.2	>20	5.5 ± 0.9	35 ± 1.7	2.3 ± 0.1
25I-NBOMe	0.37 ± 0.02	0.32 ± 0.01	6.7 ± 1.1	0.90 ± 0.13	2.1 ± 0.2	0.09 ± 0.01	0.44 ± 0.07	4.0 ± 0.8	1.3 ± 0.5	5.4 ± 0.5	1.0 ± 0.2
25N-NBOMe	0.85 ± 0.11	0.59 ± 0.07	18 ± 6.7	2.4 ± 0.1	4.5 ± 0.8	0.21 ± 0.04	2.2 ± 0.1	>20	7.2 ± 0.5	13 ± 1.2	5.1 ± 0.3
25P-NBOMe	0.31 ± 0.08	0.41 ± 0.07	3.1 ± 0.1	0.87 ± 0.08	2.3 ± 0.3	1.7 ± 0.2	0.06 ± 0.01	0.24 ± 0.03	2.8 ± 0.3	4.7 ± 0.4	5.2 ± 0.4
25T2-NBOMe	0.55 ± 0.17	0.45 ± 0.04	7.7 ± 0.4	1.6 ± 0.3	3.0 ± 0.4	0.49 ± 0.04	0.35 ± 0.02	4.2 ± 0.6	5.9 ± 0.4	8.6 ± 1.8	5.0 ± 0.2
25T4-NBOMe	0.58 ± 0.25	0.26 ± 0.03	4.9 ± 0.5	1.7 ± 0.5	1.9 ± 0.3	5.4 ± 0.3	0.12 ± 0.02	1.6 ± 0.4	4.3 ± 0.8	6.2 ± 1.5	8.1 ± 0.3
25T7-NBOMe	0.34 ± 0.06	0.36 ± 0.02	4.1 ± 0.2	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	0.09 ± 0.03	1.0 ± 0.2	3.7 ± 1.1	4.8 ± 1.4	3.2 ± 0.2
Mescaline-NBOMe	3.0 ± 1.2	0.81 ± 0.05	>14	9.6 ± 2.6	>17	14 ± 1.2	13 ± 5.6	>20	46 ± 7.5	>30	24 ± 1.3
LSD	0.67 ± 0.18	0.012 ± 0.002	0.31 ± 0.1	0.025 ± 0.0004	0.096 ± 0.005	1.1 ± 0.2	0.45 ± 0.05	10 ± 2.9	>30	>30	>30

[a] Values are K_i given as μM (mean ± SD). Comparative Ki values for known monoamine transporter inhibitors were: 0.015 ± 0.01 μM for reboxetine at the NET.
[b] 0.06 ± 0.01 μM for methylphenidate at the DAT.
[c] 0.005 ± 0.001 μM for citalopram at the SERT.

Table 3
Monoamine transporter inhibition.

	NET	DAT	SERT
	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)
2C-series			
2C-B	44 (33–58)	231 (196–271)	18 (12–27)
2C-C	93 (64–137)	305 (243–383)	74 (58–95)
2C-D	45 (28–72)	626 (536–730)	77 (60–98)
2C-E	26 (18–37)	275 (221–343)	62 (52–74)
2C-H	125 (97–161)	857 (752–976)	311 (238–408)
2C-I	22 (16–31)	126 (103–155)	13 (10–16)
2C-N	287 (223–369)	>900	154 (112–213)
2C-P	94 (73–120)	198 (136–287)	30 (22–41)
2C-T-2	153 (152–154)	332 (332–332)	62 (62–62)
2C-T-4	134 (92–195)	294 (242–357)	113 (92–138)
2C-T-7	135 (115–163)	261 (210–324)	44 (36–52)
Mescaline	>900	841 (590–1200)	367 (291–462)
N-benzylphenylethylamines (NBOMes)			
25B-NBOMe	6.7 (5.6–8.1)	117 (89–154)	7.1 (5.7–8.8)
25C-NBOMe	5.9 (4.4–7.8)	70 (56–87)	7.3 (5.6–9.6)
25D-NBOMe	4.0 (3.0–5.3)	106 (81–140)	3.9 (2.6–5.7)
25E-NBOMe	11 (8.3–14)	100 (88–112)	8.3 (6.2–11)
25H-NBOMe	10 (7.8–13)	120 (101–144)	12 (9.7–14)
25I-NBOMe	10 (7.4–14)	65 (46–89)	6.8 (4.8–9.5)
25N-NBOMe	33 (25–44)	245 (194–310)	20 (15–26)
25P-NBOMe	14 (11–16)	82 (61–110)	12 (9.3–16)
25T2-NBOMe	25 (15–42)	67 (54–84)	20 (14–29)
25T4-NBOMe	28 (22–35)	58 (43–80)	14 (11–18)
25T7-NBOMe	34 (29–40)	55 (45–68)	17 (13–23)
Mescaline-NBOMe	89 (61–130)	449 (303–665)	85 (63–116)
LSD	>900	>900	>900
Monoamine transporter inhibitors			
Reboxetine	0.036 (0.030–0.044)	ns	ns
Methylphenidate	ns	0.12 (0.09–0.16)	ns
Citalopram	ns	ns	0.045 (0.037–0.057)

Values are means of three to four independent experiments and 95% confidence intervals (CI). ns, not shown.

4. Discussion

We pharmacologically characterized the *in vitro* receptor interaction profiles of novel recreationally abused hallucinogenic *N*-2-methoxybenzyl-substituted phenethylamines compared with their 2C phenethylamine analogs. Both the NBOMe and 2C drugs potently interacted with serotonin 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors and $TAAR_{1rat}$. We also found several consistent and potentially important structure-affinity relationships for the NBOMe drugs, their 2C analogs, and several targets. Specifically, *N*-2-methoxybenzyl substitution increased the binding affinity for and/or activation potency at serotonergic 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors, adrenergic α_1 receptors, dopaminergic $D_{1\text{-}3}$ receptors, histaminergic H_1 receptors, and monoamine transporters but reduced binding to 5-HT_{1A} receptors and $TAAR_1$.

The 5-HT_{2A} receptor mediates hallucinogenic drug properties (Halberstadt and Geyer, 2011; Nelson et al., 1999; Nichols, 2004; Vollenweider et al., 1998) and is therefore considered the key target of hallucinogenic phenethylamines, including 2C and NBOMe drugs (Braden et al., 2006; Halberstadt, 2015; Halberstadt and Geyer, 2014). *N*-2-methoxybenzyl substitution consistently increased the already high *in vitro* affinity of 2C drugs for 5-HT_{2A} receptors, in agreement with data on 25H-NBOMe and 25I-NBOMe *vs*. 2C-H and 2C-I, respectively (Braden et al., 2006; Heim, 2004). All of the NBOMe drugs exhibited low nanomolar or even sub-nanomolar affinity for 5-HT_{2A} receptors, confirming studies on 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOMe, and 25B-NBOMe that used rat receptors (Braden et al., 2006; Ettrup et al., 2011, 2010; Nichols et al., 2015) or human receptors (Braden et al., 2006; Hansen et al., 2014; Nichols et al., 2015). Generally, 5-HT_{2A} receptor affinity correlates with hallucinogenic drug potency in humans (Halberstadt, 2015; Titeler et al., 1988), and NBOMe drugs can be expected to be extremely potent hallucinogens *in vivo*. Indeed, higher incidences of hallucinations and delusions have been reported in patients with NBOMe compared with 2C drug intoxication (Forrester, 2013, 2014; Srisuma et al., 2015).

Surprisingly, the consistent six-to 100-fold increase in 5-HT_{2A} receptor affinity that was produced by *N*-2-methoxybenzyl substitution did not translate into a similar increase in 5-HT_{2A} receptor activation potency, and the activation efficacy was even reduced compared with the 2C drugs in our functional assay. In contrast, others found that *N*-2-methoxybenzyl substitution in 2C-H or 2C-I increased the potency for rat or human 5-HT_{2A} receptor activation in the inositol phosphate hydrolysis assay *in vitro* (Braden et al., 2006). However, high-affinity agonist binding does not correlate well with inositol phosphate turnover (Acuna-Castillo et al., 2002; Roth et al., 1997), suggesting that additional ligand–receptor interactions contribute to receptor activation (Halberstadt, 2015; Nichols, 2004). Additionally, marked discrepancies between inositol phosphate hydrolysis activation and other *in vitro* assays and the *in vivo* effects of hallucinogens in laboratory animals or humans are well recognized (Nichols, 2004; Saez et al., 1994; Villalobos et al., 2004). Thus, although most of the effects of hallucinogens are clearly mediated by 5-HT_{2A} receptor activation (Halberstadt, 2015; Nichols, 2004), the signaling pathways that mediate these effects have not yet been conclusively identified (Halberstadt, 2015).

Currently unknown pharmacokinetic characteristics of NBOMe drugs may also influence drug potency *in vivo*. For example, differences in the *in vivo* brain binding properties of *N*-2-methoxybenzyl-substituted positron emission tomography tracers were reported for substances with similar *in vitro* 5-HT_{2A} receptor binding properties (Ettrup et al., 2011). Most importantly, NBOMe drugs are used recreationally at higher doses than LSD (Bersani et al., 2014; Halberstadt and Geyer, 2014), despite their higher 5-HT_{2A} receptor binding affinities. The lower *in vivo* potency of orally administered NBOMe drugs could be explained by their lower hepatic stability that reduced oral bioavailability compared with 2C drugs (Leth-Petersen et al., 2014). Thus, high 5-HT_{2A} receptor binding or activation *in vitro* is only one factor that potentially predicts hallucinogen potency *in vivo*. In the first *in vivo* studies that evaluated NBOMe drugs in mice, 25I-NBOMe was 14-times more potent than its analog 2C-I in inducing 5-HT_{2A} receptor-mediated head-twitch responses (Halberstadt and Geyer, 2014), consistent with the higher 5-HT_{2A} receptor binding in the present study. In contrast, 25I-NBOMe was slightly less potent in inducing head twitches than expected, based on its high 5-HT_2 binding potency (Nichols et al., 2015) and compared with LSD (Halberstadt and Geyer, 2013, 2014), consistent with the similar 5-HT_{2A} receptor activation potency of the two compounds in the present study but not reflecting the higher receptor binding potency of 25I-NBOMe compared with LSD. Additionally, 2-([2-(4-cyano-2,5-dimethoxyphenyl)ethylamino]-methyl)phenol (25CN-NBOH), which is structurally similar to the NBOMe drugs that were tested in the present study, was a more potent 5-HT_{2A} receptor agonist than 2,5-dimethoxy-4-iodoamphetamine (DOI) *in vitro* (Hansen et al., 2014) but less effective in inducing head-twitch responses in mice (Fantegrossi et al., 2015). Thus, more *in vivo* studies are needed to determine the *in vivo* potency of novel NBOMe drugs.

Within the 2C or NBOMe drug series, para-phenyl substitutions compared with 2C-H or 25H-NBOMe, respectively, enhanced 5-HT_2 receptor binding and activation potency, which was expected based on previous studies (Blaazer et al., 2008; Eshleman et al., 2014; Hansen et al., 2014; Shulgin and Shulgin, 1991). Interestingly, 5-HT_{2A} receptor activation potency increased with the size of the 4-substituent (2C-D < 2C-E < 2C-P) within the 2C series (Blaazer et al., 2008; Eshleman et al., 2014), whereas it decreased within the NBOMe series (25D-NBOMe > 25-E-NBOMe > 25P-NBOMe). Similarly, activation potency increased with halogen size for the 4-halogen-substituted 2C drugs (2C-C < 2C-B < 2C-I) but not consistently for the NBOMe analogs. Thus, *N*-2-methoxybenzyl substitution interacted with 4-phenyl substitution to affect 5-HT_{2A} receptor activation potency.

In the present study, all of the compounds were partial agonists at 5-HT_{2A} receptors, but receptor activation efficacy was consistently decreased for the *N*-2-methoxybenzyl-substituted compounds in the assay used in the present study. The high 5-HT_{2A} receptor affinity and reduction of partial activation efficacy of the NBOMe drugs suggest 5-HT_{2A} antagonistic properties of these compounds, as similarly described for LSD (Nichols, 2004). In fact, 2C drugs have been shown to act as 5-HT_{2A} receptor antagonists that inhibit 5-HT-induced currents in *Xenopus laevis* oocytes (Villalobos et al., 2004). Therefore, 5-HT_{2A} receptor antagonism has been suggested to also play a role in the mechanism of action of hallucinogens (Villalobos et al., 2004). Alternatively, other receptors, such as 5-HT_{2C} and 5-HT_1 receptors, may contribute to the mechanism of action of hallucinogens, or signaling pathways other than inositol phosphate hydrolysis may be involved (Nichols, 2004). Consistently, *N*-2-methoxybenzyl substitution increased binding affinity for 5-HT_{2C} receptors. All of the NBOMe drugs very potently bound to 5-HT_{2C} receptors, with only low (five-to 26-fold) selectivity for 5-HT_{2A} receptors over 5-HT_{2C} receptors in the binding assay, as previously shown for some NBOMe drugs (Ettrup et al., 2010; Hansen et al., 2014) and generally observed with hallucinogenic phenethylamines (Eshleman et al., 2014; Glennon et al., 1992). *N*-2-methoxybenzyl substitution only slightly increased 5-HT_{2A} over 5-HT_{2C} receptor binding selectivity. In contrast, *N*-2-methoxybenzyl substitution consistently decreased 5-HT_{1A} receptor binding, thus markedly altering 5-HT_{1A} over 5-HT_{2A} receptor binding ratios for the NBOMe drugs compared with the 2C drugs.

Thus, NBOMe drugs are unlike LSD, which is a potent 5-HT_{1A} receptor ligand and full agonist at 5-HT_{1A} receptors (Nichols, 2004). Importantly, 5-HT_{1A} receptors have been shown to contribute to the discriminative stimulus effects of some hallucinogens (Halberstadt, 2015; Nichols, 2004). Additionally, 5-HT_{1A} antagonism markedly enhanced the hallucinogenic effects of DMT in humans (Strassman, 1996). Accordingly, 5-HT_{1A} receptor stimulation has been hypothesized to counteract hallucinogenic activity (Halberstadt and Geyer, 2011; Nichols, 2004), and lower 5-HT_{1A} receptor stimulation for the NBOMe drugs may further enhance their hallucinogenic drug properties. *N*-2-methoxybenzyl substitution increased 5-HT_{2B} activation, but this is likely not relevant for the psychotropic properties of the NBOMe drugs (Blaazer et al., 2008). However, 5-HT_{2B} receptors have been implicated in substance-induced heart valve fibrosis (Bhattacharyya et al., 2009; Setola et al., 2003), and the 2C and NBOMe drugs may therefore have cardiac toxicity if used chronically.

Because NBOMe drugs produce marked sympathomimetic cardiovascular effects in humans (Wood et al., 2015), we tested whether these drugs interact with monoamine transporters similarly to cocaine or amphetamines (Simmler et al., 2013, 2014a) and other novel psychoactive substances (Rickli et al., 2015a, 2015b; Simmler et al., 2014a; Simmler et al., 2014b). *N*-2-methoxybenzyl substitution enhanced monoamine transporter inhibition compared with the 2C drugs. However, the potency of even the most potent NBOMe drugs at the NET and SERT was low and only in the 5–10 μM range, indicating that amphetamine-type monoamine transporter interactions contribute only little to the cardiostimulant effects of NBOMe drugs.

In addition to their very high 5-HT_{2A} binding affinity, we found that the NBOMe drugs and LSD had high binding affinity for adrenergic α_{1A} receptors. 2C drugs have been shown to contract blood vessels (Saez et al., 1994) through direct interactions with serotonergic 5-HT_2 and adrenergic α_1 receptors (Lobos et al., 1992). The vasoconstrictive potency of 2C drugs does not appear to correlate well with hallucinogenic potency in humans (Saez et al., 1994) or 5-HT_{2A} receptor activation. For example, 2C-D had higher affinity for 5-HT_{2A} receptors compared with 2C-H in the present study but lower potency in contracting the rat aorta (Saez et al., 1994). Additionally, 2C-N, which exhibited high affinity for 5-HT_{2A} receptors but not α_1 receptors in the present study, did not present vasoconstrictive activity (Saez et al., 1994). These findings and the relatively high affinity of the NBOMe drugs for adrenergic α_1 receptors indicate that these receptors might contribute to the stimulant-type cardiovascular effects that are typically seen in cases of NBOMe drug intoxication (Srisuma et al., 2015; Wood et al., 2015). Additionally, the behavioral effects of 25I-NBOMe in mice showed a rapid peak (within minutes), whereas the response to 2C-I was relatively flat (Halberstadt and Geyer, 2014). Thus, such substance characteristics as the higher lipophilicity of NBOMe drugs may further accentuate the clinical drug response. As a result, there is likely a high risk of overdose with NBOMe drugs, and several fatalities have been reported (Hill et al., 2013; Srisuma et al., 2015; Walterscheid et al., 2014; Wood et al., 2015).

Both the 2C and NBOMe drugs bound to $TAAR_1$, with few exceptions. *N*-2-methoxybenzyl substitution slightly decreased $TAAR_1$ binding affinity as previously shown for other N-substitutions in phenethylamines (Lewin et al., 2008). $TAAR_1$ modulates psychotropic drug actions. Importantly, methylenedioxymethamphetamine inhibits its own stimulant effects via $TAAR_1$ activation (Di Cara et al., 2011). Whether similar $TAAR_1$-mediated "auto-inhibition" exists for hallucinogens remains to be determined. One hypothesis is that the lower $TAAR_1$ activity that is associated with *N*-2-methoxybenzyl substitution may also enhance psychostimulant drug properties *in vivo*.

LSD exhibited high affinity for D_1, D_2 and D_3 receptors, as previously shown (Watts et al., 1995) and in contrast to phenethylamines. D_2 receptors have been shown to contribute to the interoceptive effects of LSD in rats (Halberstadt and Geyer, 2013, 2014). Although *N*-2-methoxybenzyl substitution increased $D_{1\text{-}3}$ receptor binding affinity compared with 2C drugs, NBOMe drugs were less potent at $D_{1\text{-}3}$ receptors compared with LSD, indicating that LSD has a unique mixed dopaminergic-serotonergic binding profile.

In summary, NBOMe drugs are highly potent 5-HT_{2A} receptor ligands and partial 5-HT_{2A} receptor agonists, similar to the classic hallucinogen LSD, but with 5-HT_2 over 5-HT_1 receptor selectivity, unlike LSD. NBOMe drugs bind to adrenergic α_1 receptors and $TAAR_1$, similar to LSD, but do not bind to dopaminergic $D_{1\text{-}3}$ receptors, unlike LSD. The *in vitro* binding profiles of NBOMe drugs suggest that they have higher hallucinogenic effects and potency compared with their parent 2C drugs and are similar to the very potent hallucinogen LSD because of their similar or even higher potency at 5-HT_{2A} receptors. At higher doses, NBOMe drugs may also exhibit additional stimulant properties through α_1 receptor interactions.

Conflicts of interest

M.C.H. is an employee of F. Hoffmann-La Roche.

Authorship contributions

Participated in research design: Rickli, Liechti.
Conducted experiments: Rickli, Luethi, Reinisch, Buchy.
Performed data analysis: Rickli, Hoener, Liechti.
Wrote or contributed to the writing of the manuscript: Rickli, Liechti.

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Discussion

This thesis presents the *in vitro* pharmacological profiles of 75 novel psychoactive substances including classical drugs like MDMA, amphetamine, methamphetamine, psilocin, mescaline, and LSD. We found a broad spectrum of interactions of these compounds with relevant brain monoaminergic target sites. Broadly, we could distinguish two main groups by their way of action: 1. Compounds primarily interacting with monoamine transporters (Simmler et al. 2014, Simmler et al. 2014, Rickli et al. 2015) and 2. Phenethylamine derivatives, which were potent serotonin 5-HT_{2A} agonists with no or only weak serotonin and/or norepinephrine reuptake inhibitors (Rickli et al. 2015). Most of the monoamine uptake inhibitors inhibited the NET in low micromolar concentrations. The clinical characteristics of a compound (MDMA-like or stimulant-like) were mostly due to different potencies inhibiting the dopamine vs. serotonin reuptake, reflected in the DAT/SERT inhibition ratio presented in Figure 3.

The most serotonergic over dopaminergic drugs were trifluoromethylpiperazie (TFMPP), 5-(2-aminopropyl)2,3-dihydrobenzofuran (5-APDB), 4-methylthioamphetamine (4-MTA), 1-(2,3-dihydrobenzoduran-5-yl)-N-methylpropan-2-amine (5-MAPDB), para-methoxyamphetamine (PMA), para-methoxy-N-methylamphetamine (PMMA), meta-chorpheylpiperazine (m-CPP), 5-(2-aminopropyl)benzofuran (5-APB), 6-(2-aminopropyl)-2,3-dihydrobenzofuran (6-APDB), and MDMA all with a DAT/SERT inhibition ratio below 0.1. In fact, these substances are all reportedly relatively similar to MDMA with regards to their clinical toxicology. On the other hand, alpha-pyrrolidinopentiophenone (α-PVP), methylphenidate, and desoxypipradrol (2-DPMP), and pyrovalerone-cathinones were the most dopaminergic compounds in this series of tested substances, with high DAT/SERT inhibition ratios (>100). Several *in vitro* studies with rats and mice indicated that the pyrovalerones α-PVP and MDPV show high reinforcing properties and clinical reports indicate a high abuse and addiction potential in humans (Karlsson et al. 2014, Watterson et al. 2014, Watterson et al. 2014, Aarde et al. 2015, Karila et al. 2015).

In contrast to the pronounced differences in DAT and SERT inhibition potencies, nearly all tested monoamine uptake inhibitors potently inhibited the NET with IC_{50} values below 1 µM as also found in earlier studies (Eshleman et al. 2013, Simmler et

al. 2013). Though, all NPS have sympathomimetic activation in common and these appear to correlate with the recreational doses used (Eshleman et al. 2013).

However, many NPS also possess transporter substrate properties leading to substance-induced transporter-mediated monoamine release, similar to MDMA and other amphetamines (Baumann et al. 2012, Baumann et al. 2013, Eshleman et al. 2013, Simmler et al. 2013, Marusich et al. 2014, Saha et al. 2015).

We found that many of the MDMA-relatives possessed substrate properties for at least one of the monoamine transporters. Triple monoamine releaser (similar to MDMA, methamphetamine, and amphetamine) were, PMA, PMMA, N-ethylamphetamine, 3-fluoromethcathinone (3-FMC), 4-fluoroephedrine (4-FEP), 4-fluoroamphetamine (4-FA), 4-fluoromethamphetamine (4-FMA), 4-ethylmethcathinone (4-EMC), 4-methylmethcathinone (mephedrone), 5-APB, 6-APDB, 4-(2-Aminopropyl)benzofuran (4-APB), and 3,4-methylenedioxyamphetamine (MDA).

Most cathinones (excl. pyrovalerone cathinones), were substrates of the DAT and induced DA release, whereas DA release was not significant in our assay with methedrone, ethcathinone, buphedrone, pentedrone, and N,N-dimethylcathinone.

Pipradrol structures (diphenylprolinol (D2PM), desoxypipradrol (2-DPMP), and methylphenidate) and the pyrovalerone-cathinones (3,4-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP), 3,4-methylenedioxy-α-pyrrolidinobutiophenone MDPBP, methylenedioxypyrovalerone (MDPV), naphyrone, α-PVP, and pyrovalerone) did not release monoamines at any of the three transporters together with pentedrone, N,N-dimethylcathinone, m-CPP, TFMPP, and the benzodifuran 2C-B-FLY. Probably, the pyrovalerone-, piperazine-, piperidine- and pyrrolidine structures prevented uptake as transporter substrate. These results are in line with previous studies, at least described for pyrovalerone-cathinones (Marusich et al. 2014), lacking substrate properties in contrast to simple ring-substituted cathinones (Simmler et al. 2013). Also the enlargement of the alpha-methyl group to alpha-ethyl or alpha-propyl may possibly also abolish substrate characteristics.

In contrast to the above discussed monoamine transporter blockers and releasers, 2C drugs and in particular their NBOMe (N-2-methoxybenzyl)-substituted counterparts, showed high affinity at nanomolar concentrations to the serotonin 5-HT_{2A} receptor. This is a very exciting finding, because NBOMes showed even lower

K_i values than LSD (K_i: 0.0042 µM), which was known so far as one of the most potent hallucinogenic compound (Braden et al. 2006, Passie et al. 2008, Ettrup et al. 2011, Hansen et al. 2014, Nichols et al. 2015). However, these compounds had similar activation potencies to LSD and were partial agonists like LSD. This was in contrast to the structurally different benzodifuran 2C-B-FLY, which was also a highly potent 5-HT_{2A} agonist with nearly full agonist properties (K_i: 0.011 µM; activating efficacy: 82 %) unlike LSD (Rickli et al. 2015).

We also found NPS, which interacted with the SERT and the 5-HT_{2A} receptor, with characteristics similar to both, MDMA and LSD. Such mixed compounds were 5-iodo-2-aminoindane (5-IAI), m-CPP, TFMPP, 5-APB, 6-(2-aminopropyl)benzofuran (6-APB), 4-APB, and 7-(2-Aminopropyl)benzofuran (7-APB), all with K_i values < 1µM for the 5-HT_{2A} receptors. Thus hallucinogenic effects are possible in addition to their MDMA-type effects, as described at least for 5-IAI, 5-APB and the trifluoromethylphenylpiperazine-benzylpiperazine (TFMPP-BZP) combination (Coppola et al. 2013, Simmler et al. 2014, McIntyre et al. 2015, Rickli et al. 2015). However, intoxications with the catecholamine uptake inhibitor D2PM including hallucinogenic-like effects, demonstrate that also less potent serotonin 5-HT_{2A} receptor agonists have the potential for this side effect if consumed in high doses (Wood et al. 2012).

Although most effects of the described designer drugs are mediated either over interaction with the monoamine transporters SERT, NET, and DAT, or serotonin receptors, most investigated structures also displayed affinity around 1 µM at the $TAAR_{1rat}$ with 2-(2,5-dimethoxy-4-propylphenyl)ethanamine (2C-P) and 2C-B-FLY showing lowest K_i values (0.02 and 0.03 µM), respectively. This is an interesting finding because $TAAR_1$ may serve as a promising target to treat drug addiction (Jing et al. 2015).

Nearly all of the tested designer drugs have sympathomimetic effects through the indirect increase of catechoalmines and serotonin via direct stimulation of serotonin receptors 5-HT_{2A}, and also 5-HT_{1A}, 5-HT_{2C} and 5-HT_{2B}, which is probably involved in cardiotoxic effects (Rothman et al. 2009).

Additionally, 4-fluoromethcathinone, 4-methylmethcathinone, NBOMes, and LSD also directly bind to $alpha_{1A}$ receptors in low micromolar concentrations, enhancing cardiovascular stimulation (Piascik et al. 2001, Schmid et al. 2015).

The clinical interaction study with MDMA produced empathogenic effects. MDMA significantly increased subjective feelings of “any drug effect”, “good drug effect”, “drug high”, “drug liking”, and “stimulated”. Somatic side effects included increases in body temperature, heart rate, systolic and diastolic blood pressure, and mydriasis. These effects were transient and manageable in a controlled setting but may easily get out of control, in overdose situations, drug-mixing, and/or under hot party conditions with inadequate cooling (Liechti et al. 2005, Liakoni et al. 2015).

Final remarks

The pharmacological profiles of NPS *in vitro* give a first important picture of the pharmacodynamics of these compounds and predictions of the psychotropic and somatic effects *in vivo*. Additional case reports from NPS users enlarge the picture of a certain drug, which also gives an idea about the effective doses of a compound. Nevertheless, small modifications in the structure of the substance may lead to relevant pharmacologic changes with an additional unknown risk of acute and long-term toxicity.

Additional evaluation of pharmacokinetic parameters would help for a better understanding of psychoactive compounds.

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