Pharmacology of Amphetamine-Type Designer Drugs

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„SCHÜLER. Ich wünschte, recht gelehrt zu werden,
Und möchte gern, was auf der Erden
Und in dem Himmel ist, erfassen,
Die Wissenschaft und die Natur.
MEPHISTOPHELES. Da seid Ihr auf der rechten Spur;
Doch müsst Ihr Euch nicht zerstreuen lassen.
SCHÜLER. Ich bin dabei mit Seel' und Leib;
Doch freilich würde mir behangen
Ein wenig Freiheit und Zeitvertreib
An schönen Sommerfeiertagen."

Aus Goethes Faust

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Summary

In this thesis project, we studied the pharmacology of long-known psychostimulants and of novel amphetamine-type designer drugs in vitro. On the basis of the pharmacological characterization, we focused on the drug effects in humans, investigating the pharmacological effects of the widely used amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy"). Furthermore, we investigated potential antagonistic treatments for methamphetamine dependence.

In vitro, we characterized the pharmacology of a comprehensive set of drugs, determining their action at targets found in monoaminergic synapses. We used HEK-293 cells transfected with the respective genes for the three monoamine reuptake transporters and post-synaptic receptors. We performed pharmacological assays with radiolabeled substrates or ligands to determine drugs' transport modulation properties and receptor affinities. We classified novel designer drugs that were cathinone-derivatives according to their pharmacology and discriminated between three groups. Cocaine-MDMA-mixed cathinones were drugs that inhibited the dopamine (DA) and serotonin (5-HT) uptake with equal potencies to cocaine, but preferentially released transporter-mediated 5-HT like MDMA. Methamphetamine-like cathinones inhibited DA uptake preferentially over 5-HT uptake and released DA, comparable to methamphetamine. Pyrovalerone-cathinones were extremely potent DA uptake inhibitors, but non-releasers. Additionally, we found methedrone as only serotonergic cathinone, similar to MDMA and other *para*-methoxylated amphetamines. We described key findings upon blood-brain-barrier characteristics and differences between β-ketonated and non-β-ketonated amphetamines in receptor binding and DA/5-HT preferences. All amphetamine-type drugs inhibited norepinephrine (NE) uptake, which indicated the importance of the norepinephrine system as contributor to the effects of psychostimulants.

In humans, we assessed the role of NE and 5-HT in the mechanism of action of MDMA in two placebo-controlled, double-blind, crossover studies. To elucidate the contribution of the respective monoamines, we attenuated the MDMA-induced psychotropic and cardiovascular effects with the NE-transporter (NET) inhibitor

reboxetine and also with the mixed 5-HT transporter (SERT)/NET inhibitor duloxetine. We found that 5-HT primarily mediates empathogenic mood effects whereas NE is primarily involved in the MDMA's cardiostimulant effects. PK-PD relationships combining subjective- or cardiostimulant effects with MDMA's pharmacokinetics in relation to time depicted the tolerance of the drug's effects. Predicting drug effects with in vitro 5-HT and NE release characteristics, we highlighted the role of NE in MDMA's mode of action.

We also showed that 5-HT and/or NE play a role in the MDMA-induced inappropriate secretion of antidiuretic hormone, which is involved in the development of potentially serious complications such as brain edema evolving from hyponatremia. In one of the aforesaid clinical studies, duloxetine attenuated the MDMA-induced increase of antidiuretic hormone, which was more distinctive in female than male subjects.

Finally, we addressed our research to the possibilities of pharmacological modulation of amphetamine-type drug dependence by antagonizing DA-related effects. We assessed in vitro the potencies of three DA transporter (DAT) inhibitors to block methamphetamine-induced DA release, which is a known mechanism for drug dependence. The DA uptake inhibition potencies of the three assessed drugs correlated linearly with their potencies to inhibit methamphetamine-induced DA release. The strong action on the DA system and the associated abuse potential of the most potent release blocker, 3,4-methylenedioxypyrovalerone, depicts the difficulty to find unproblematic pharmacological treatment for drug dependence.

Taken together, we characterized 5-HT, NE, and DA in amphetamine-type drug action in vitro and also illustrated their relevance for drugs' effects in humans.

Introduction

1. Designer drugs - definitions

Designer drugs are, to date, an unclear and opulent accumulation of different substances, which are ingested weekend for weekend all over the world in order to change one's state of perception or to party all night. To look for an exact definition of the term *designer drug* is almost as confusing as trying to get an overview over the different molecules that are available as designer drugs.

Designer drugs are substances that were designed from the example of illegal drugs by slight modification of the molecule in order to obtain uncontrolled substances with similar effects than the controlled exemplary drugs [1]. This is the definition of the authority that surveys the European illegal drug market, namely the European Monitoring Center for Drugs and Drug Addiction (EMCDDA). German Wikipedia is consistent with the EMCDDA definition and points out that the key feature of a *designer drug* is its design for narcotic drug purpose, which would not include substances such as amphetamine or MDMA, as they were first designed for medical purposes [2]. The Meyer's encyclopedia is more general in their definition. It explains designer drugs as "synthetic narcotic drugs, chemical derivatives of known addictive drugs" and gives Ecstasy as example [3]. Sometimes *designer drugs* is used as synonym for *synthetic drugs*, which includes all drugs synthetically and not naturally derived [2]. Due to the inconsistency of definitions, I take here the freedom to define the term *designer drug* in our way of understanding. We understand *designer drugs* in this thesis as substances that were synthetically produced and currently abused as stimulant and/or recreational drugs.

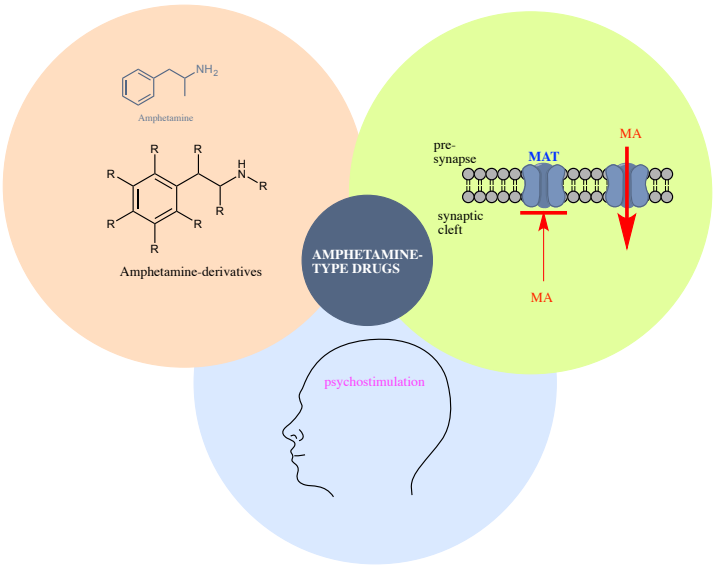

Figure 1: Graphical definition of the term *amphetamine-type drugs* as it is referred to in this thesis. Amphetamine-type drugs unify structural similarities (upper left circle), similar molecular mechanism of action (upper right circle), and comparable psychostimulant effects in humans (lower circle). MA = monoamine, MAT = MA-transporter.

The term *amphetamine-type drugs* or shortly *amphetamines*, is use here for substances that resemble amphetamine in their chemical structure, in their molecular mechanism of action, and in the experienced effects in drug users. Derivatives of amphetamine can be derived from modification on the benzene ring, the amino group, and the α- or β-position of the carbon chain. A key characteristic of amphetamine pharmacology is the induction of monoamine release mediated by their respective uptake transporters [4]. Also, amphetamine inhibits reuptake of the monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) [5]. Characteristic psychotropic effects of amphetamines are euphoria and stimulation, but no hallucinations at the doses typically used [6, 7].

2. Novel designer drugs

In chapter 1 of this thesis, our research is strongly focused on novel amphetamine-type designer drugs. It is therefore worth to say some words about this kind of novel drugs. Within the last few years there was a massive increase in the diversity of available designer drugs (see Figure 2). Since many of these designer drugs were, or

still are, uncontrolled substances, they are also known as “legal highs” [8]. Mephedrone (also “Meow”, “MCAT”, “bath salt”, “research chemical”) is one of the most popular examples of novel designer drugs, which was set under control in the UK in April 2010 [9]. Many other substances followed to be controlled, while new structures emerged. In order to react quickly to newly emerged designer drugs, Swiss legislation for example, now allows for scheduling substances several times per annum into *Verzeichnis e*, which contained in December 2012 seven substance classes including cathinones and 52 single substances [10]. Media reports about “zombie drugs” in summer 2012 pointed out the relevance and actuality for research on this topic. These worldwide published stories associated psychotic cannibalism with the consumption of “bath salt” that most likely contained the novel designer drugs mephedrone, 3,4-methylenedioxypyrovalerone (MDPV), and/or methylone [11]. Dealing with novel designer drugs in this thesis, we mainly focus on cathinone-derivatives, which have become a very popular class of designer psychostimulants and include mephedrone, MDPV, methylone and many others. From the 40 designer drugs newly emerged on the EU illicit drug market in 2010, 15 were cathinone-derivatives [12]. Cathinones are synthetic derivatives of cathinone, which is found in *Catha edulis*. The fresh leaves of this tree, so-called Kath, are chewed in many African and Arab countries. Khat and the synthetic cathinones have stimulant amphetamine-type effects in humans [8]. Since the reported psychostimulant effects produced by cathinones are similar to those of other amphetamines like MDMA or methamphetamine, it was nearby to compare the pharmacology and mechanism of action of cathinones to these well-known and thoroughly investigated designer drugs. We assessed the novel designer drugs in vitro only, and for mechanistic studies that were partially conducted in humans we used the model substances MDMA and methamphetamine. The findings and conclusions based on the experiments with MDMA and methamphetamine in this study should also be expanded to our novel designer drugs, since the in vitro findings in chapter 1 build the bridge between the pharmacology of novel drugs and the classical stimulants, and the studies in chapter 2 and 3 correlate relevant effects in humans with in vitro characteristics.

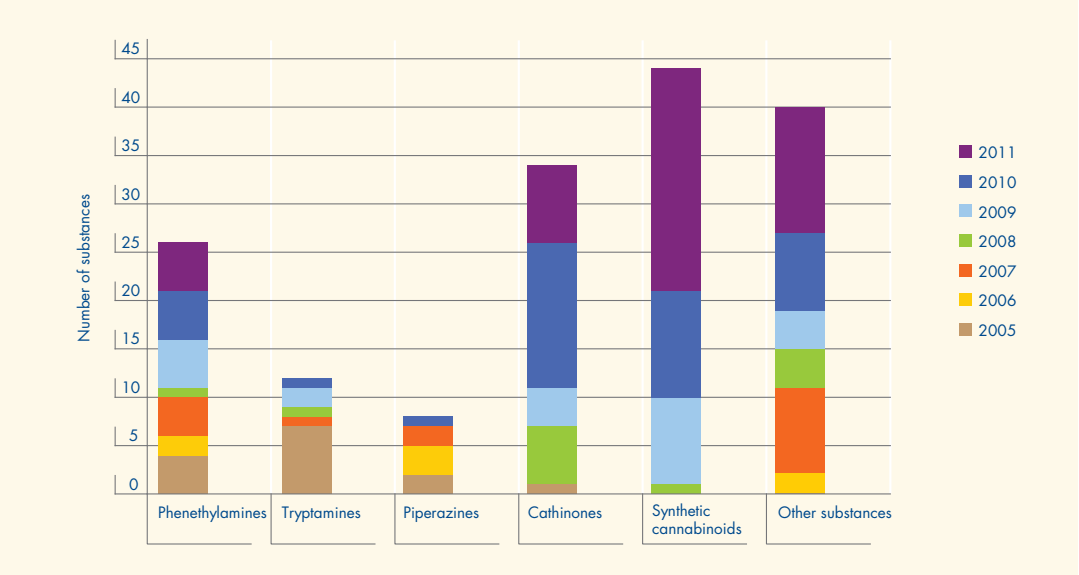

Figure 2: Number of newly detected substances sold as recreational designer drugs as presented by EMCDDA in their annual report 2012 [1]. Cathinones represent the second largest group of chemically defined substances.

3. Molecular mechanism of action of amphetamines

The molecular mechanism of action of amphetamine and its derivatives methamphetamine and MDMA is well investigated. The main site of action of amphetamine-type drugs is at the synapse of monoaminergic neurons located in the brain mainly in the reticular formation [13]. All amphetamine-type drugs inhibit DA, NE, and 5-HT reuptake from monoaminergic synapses, although with different potencies. Typically for amphetamines, they also induce reverse transport (also referred to as release or efflux) of neurotransmitter not exocytotically, but through monoamine transporters [4]. Since the reuptake transporters are the only targets that clear the monoaminergic neurotransmitters from the synapses and thus regulate signal transmission, inhibition of reuptake and induction of reverse transport result in profound changes of signal transmission [14, 15].

For the understanding of the pharmacological assays with which we assessed all drugs in this thesis, it is crucial to know the physiology of a synapse, the site where the amphetamine-type drugs act. I will describe in the following sections the localization of amphetamines' targets, the mechanism of uptake inhibition and the mechanism of drug-induced release. I will also discuss the relevance to discriminate uptake inhibition and release induction.

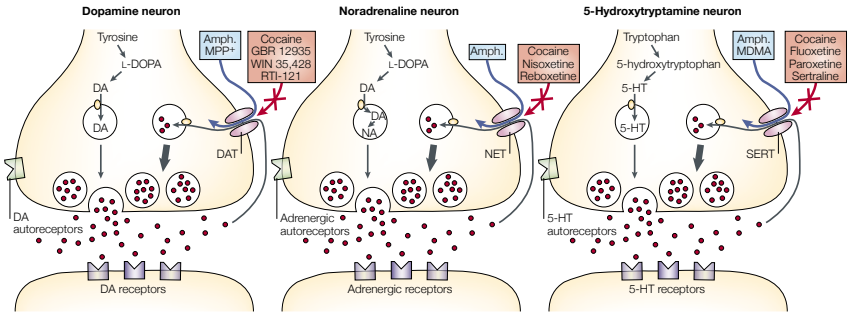

Figure 3: Schematic overview on synaptic physiology of DA, NE, and 5-HT neurons as illustrated by Torres et al. [14]. Monoaminergic neurotransmitters are synthesized in the neuron, stored in vesicles and released for signal transmission via exocytotic fusion of the storage vesicles with the membrane. To end signal transmission, which occurs upon the stimulation of post-synaptic receptors, monoamines are cleared from the synaptic cleft by reuptake via monoamine transporters. Monoamine- and vesicular transporters are targets of psychoactive therapeutics and/or psychostimulant drugs of abuse. While pure uptake inhibitors (e.g., cocaine) act extracellularly, substrate-type releasing drugs (e.g., MDMA) are transported into the presynapse and also act intracellularly.

3.1. Localization of main sites of action, brain anatomy and synaptic physiology

Brain regions containing DA neurons are the basal ganglia (includes striatum with nucleus accumbens), the ventral tegmental area, and the substantia nigra [13]. Noradrenergic neurons are mainly found in the locus coeruleus and are responsible for adrenergic neurotransmission [13]. Serotonergic neurons are located in raphe nuclei and are involved in the regulation of mood, anxiety, and perception [13]. From their origin, monoaminergic neurons expand widespread projections into different brain regions, representing the dopaminergic, noradrenergic, or serotonergic systems [16]. Dopaminergic neurons are important neurons in the brain's mesolimbic system, which is essential for reward [13].

A monoaminergic neuron physiologically releases neurotransmitter exocytotically via vesicles, in which the monoamines are stored until release. Apart from the releasing function of vesicles, they protect the neurotransmitters during storage by their low pH conditions, and in the case of NE, vesicles are the location for the final step in its synthesis [16, 17]. After having been released into the synaptic cleft, monoamines reach the postsynapse and transmit the neurological signal by binding and thereby activating or deactivating postsynaptic receptors [16]. Finally, to end the signal transmission, monoamine reuptake transporters are responsible for clearing the neurotransmitters from the synaptic cleft back to the presynapse [15]. See Figure 3 for an illustrating summary of synaptic physiology.

3.2. Monoamine transport and uptake inhibition

Monoamine transporters are secondary active transporters [4] since their driving force is an ion gradient, which is sustained by a Na^+/Cl^--ATPase [15]. The monoamine uptake transport follows Michaelis-Menten kinetics, giving the transport-specific constant K_M. K_M describes the affinity by which the transporter processes its substrate [15, 18].

A wide range of therapeutics and abused substances target monoamine transporters to inhibit monoamine reuptake with different potencies and selectivities for the respective monoamines. Classical tricyclic antidepressants target all three monoamine transporters [17], whereas selective antidepressants like fluoxetine or nisoxetine are SERT or NET selective, respectively [17]. The amphetamine-like antidepressant and smoking cessation aid bupropion exhibits more potent DAT than NET inhibition [19, 20]. The quantification of drugs' transport inhibitions, giving the aforesaid characteristics of substances, follows enzyme kinetic rules and is expressed as potency (K_i or IC_{50} values) [21]. While K_i values are assay-independent constant values, IC_{50} values vary according to assays and condition modifications. For the present thesis we determined IC_{50} values only, since considerably less experiments need to be performed for this value compared to for K_i values [21]. Although Cheng and Prussoff describe ways to calculate a K_i value from a single IC_{50} value at specific assay conditions [22], we did not calculate K_i-values since we did not determine the mode of inhibition, namely competitive or non-competitive inhibition.

3.3. Drug-induced monoamine release

Increases in neurotransmitter concentrations in the synaptic cleft can occur via the physiological release of neurotransmitters by vesicles (exocytosis) and also by reuptake inhibition, since prevention of neurotransmitter recycling increases net neurotransmitter concentrations [17]. However, when amphetamines act in the synapses, there is a third way of neurotransmitter increase. Amphetamines induce non-exocytotic neurotransmitter release, not mediated by vesicles, but by the respective reuptake transporters [4]. The precise mechanism of amphetamine-induced monoamine efflux has not yet been determined. In the next sections, I summarize three different hypothetic mechanisms of drug-induced reverse transport. All models have their limitations and are contradicting either way. Thus, note that the mechanism

of release is controversially discussed and still remains to be completely resolved [4, 23]. However, all models assume an uptake of the releasing drug into the cells. This fact is essential for the interpretation of the results in this thesis and in general for correct interpretation of in vitro data for biological or clinical relevance. It will be explained in paragraph 3.4.

3.3.1. Amphetamines are substrates of the monoamine reuptake transporters

Being substrates of the reuptake transporters like monoaminergic neurotransmitters, amphetamine-type drugs exhibit a distinct characteristic from non-releasing drugs such as cocaine or methylphenidate [4]. Several attempts have been made to quantify transport of amphetamine-like drugs through monoamine transporters. Amphetamine's high lipophilicity and its preference to penetrate the cell membrane as unprotonated molecule challenge the quantification of the drug's inward transport [23]. Sitte et al. show the Na^+/K^+-ATPase- and Na^+-dependent uptake of the two amphetamine enantiomers and tyramine. They also demonstrated the inhibition of this uptake if experiments are performed on ice, which indicates active transport, or if the transporter inhibitor cocaine is present [24]. Verrico et al. showed the uptake of MDMA by DAT, SERT, and NET and it's inhibition on ice [25].

Taken together, the experiments assessing drug transport indicate similar or identical transport mechanisms for amphetamines and the physiological substrates, the monoamine neurotransmitters. This mechanistic key feature is essential to understand the mechanism of amphetamines-induced reverse transport of monoamines [4] and to understand the toxicological relevance of a substrate versus a non-transported non-releasing uptake inhibitor.

3.3.2. Release model A: "facilitated exchange diffusion" – the revolving door model

Sitte and Freissmuth explain in their comprehensive review one widely postulated model for monoamine release [23]. The "facilitated exchange diffusion" model considers the transporter as a protein in two main conformations, the outward-facing conformation and the inward-facing conformation (see Figure 4). If the binding site faces to the synaptic cleft, the substrate (e.g., a monoaminergic neurotransmitter or

the release-inducing amphetamine) binds to the transporter together with Na^+, and the transporter changes its conformation to the binding site facing to the cytoplasma. Since Na^+ concentration on the cytoplasmatic site is low, the cotransported Na^+ molecule diffuses immediately from the binding site, resulting in a weak binding of the substrate, which then subsequently is released from its binding pocket to the cytoplasm. In the inward-facing state of the transporter, a substrate (e.g., the to be released neurotransmitter) located intracellularly can then bind to the transporter for a reverse transport from the inside to the outside. This transport occurs physiologically with a significantly lower probability since intracellular Na^+ is diminished and thus substrate-transporter binding, for which Na^+ is essential, occurs less frequently on the intracellular than on the extracellular site. However, the presence of a substrate, such as amphetamine, enhances the probability for inward-facing confirmation, and thus enhances the probability for reverse transport. Sulzer argues in his review that the facilitated exchange diffusion model follows a one to one exchange of releasing drug (substrate transported from extra- to intracellular space) with neurotransmitter (released by reverse transport from the cytosol to the synapse) [4]. Uptake transport of amphetamines or other releasing drug would be crucial to induce release and thus K_M of the uptake of the releasing drug must theoretically correlate linearly with its potency to induce release. However, release can also be induced by disruption of the ion gradient over the plasma membrane [26, 27], or if amphetamine is injected directly into a neuron and thus circumvents its transporter mediated uptake [28]. Experiments with Zn^{2+} indicate that uptake and release do not necessarily need to occur via the same target. Zn^{2+} on one hand inhibits substrate uptake and on the other hand enhances amphetamine-stimulated DA efflux [29-31]. These observations led to seek for extended or other models.

3.3.3. Release model B: “Channel-like transport modes”

In the facilitated exchange diffusion model (described in details in 3.3.2.), we assume transport of a substrate to come along with the transport of an ion and thus with charge transfer. However, the observation of charge transfer not correlating with substrate transport through the transporters [32-34] led to the proposal of a “channel-like transport mode” (Figure 4). It assumes the transporter’s confirmation to change to a structure that forms a pore or channel, allowing for autonomous diffusion of ions

and substrate [4]. This model is supported by a variety of observations. For example, patch-clamp measurements detect amphetamine-induced events that resemble classical ion-channels [28]. Sitte et al. support the channel-like transport theory in a study where they measured the uptake of the substrates D-amphetamine, L-amphetamine, tyramine, and DA [24]. Substrate uptake kinetics reveal a considerably lower K_M-value for D-amphetamine (0.8 µM) than the K_M-values of the other substrates, but V_{max} of DA was about 20 times higher than D-amphetamine's V_{max}. They then found release-inducing properties of the substrates to correlate better with their current-inducing properties, measured by patch-clamp method, than with their uptake properties. However, release via the channel-like mode of a transporter is probably a rare event compared to facilitated exchange diffusion [35].

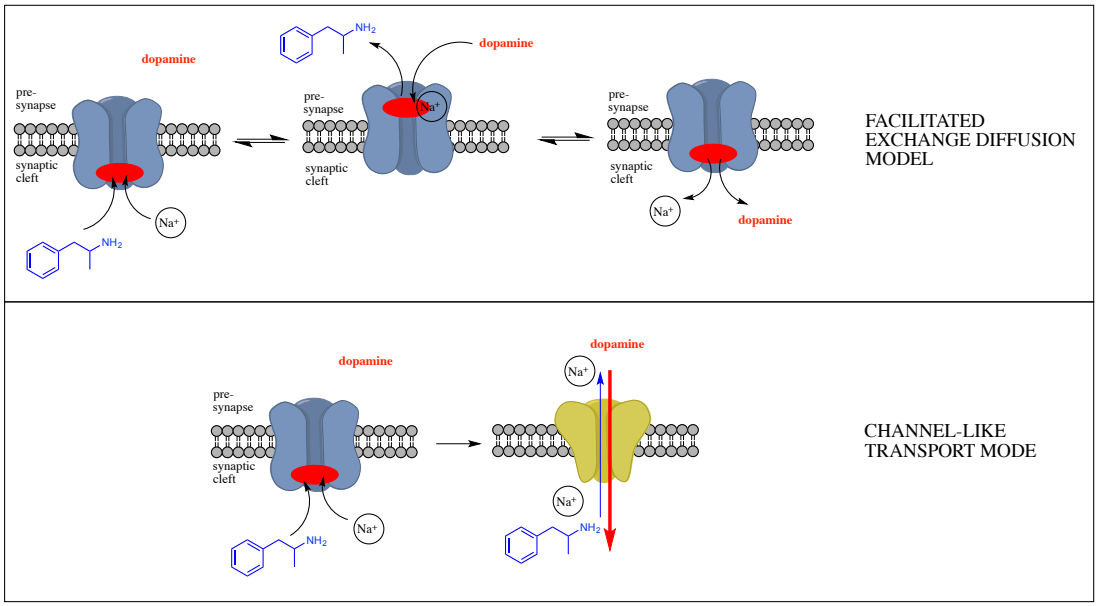

Figure 4: Schematic explanation of the channel-like transport mode and the facilitated exchange diffusion model as explained in the previous paragraphs, exemplary shown DAT (membrane protein) and action of amphetamine (chemical structure). Red indicates the binding site for amphetamine or DA. The yellow transporter visualizes DAT's structural change to a channel-like pore.

3.3.4. Release model C: "Oligomer-based counter-transport model"

On the basis of lacking complete explanation of several observations with the fascilitated exchange diffusion and channel-like transport models as described in the previous sections, Sitte and Freissmuth describe in their review a model based on the observation that reuptake transporters are expressed in the plasmamembrane as

oligomers [23]. While, at maximal release, 50% of the transporters are in their outward-facing conformation and unphosphorylated, the remaining 50% are inward-facing and phosphorylated. Cytoplasmic neurotransmitters can then bind to the inward-facing transporter and are transported into the synaptic cleft. Bell-shaped release curves with a maximal release at lower concentrations than maximally applied, illustrate this oligomeric theory [36]. The "oligomer-based counter transport model" cannot yet explain if both or one of the two transporter confirmations is channel-like or needs one-to-one Na^+ co-transport analogue to the facilitated diffusion model. Nevertheless, this model could unify the two controversially discussed models and explain phenomena, which were inexplicit before and is worth being further assessed.

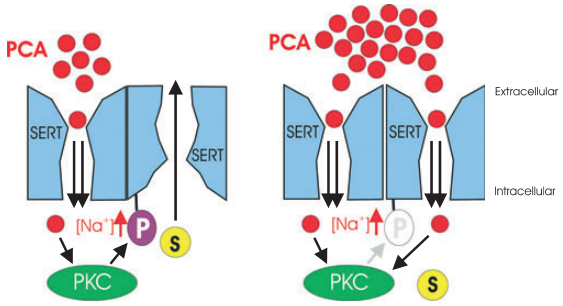

Figure 5: The oligomer-based counter-transport model explained and illustrated by Sitte and Freismuth [23]. The left oligomer (here drawn as dimer) of SERT shows maximal 5-HT release with one moiety of transporters occupied by *para*-chloroamphetamine (PCA), activating protein kinase C and allowing the other moiety of SERT to be phosphorylated. The phosphorylated transporters are able to release 5-HT from the intra- into the extracellular space. The right oligomer of SERT illustrates a situation with increased PCA concentrations, which prevents 5-HT release due to full occupation of SERT with PCA.

3.3.5. Pathways and transporter modulation

Regardless of the release models presented in the previous sections, drug-induced monoamine release through reuptake transporters occurs upon activation of signaling or modification mechanisms. DA release requires intracellular phosphorylation through protein kinase activity [37] and/or Ca^{2+}/calmodulin-dependent phosphorylation [23]. Transporter internalization results in reduced transporter surface expression and thus in reduction of the amount of proteins available for release. This internalization, probably regulated via G-protein coupled receptors, is proposed to be a regulatory mechanism for drug-induced release [38].

3.4. Toxicologically relevant distinction of non-releasing and releasing psychostimulants

Compared to pure uptake inhibitors (e.g., fluoxetine) substrate-type releasers (e.g., fenfluramine) increase synaptic neurotransmitter concentration much more pronounced [39]. Also, psychotropic effects evoked by the 5-HT releaser MDMA are abolished due to SERT blockade with the pure uptake inhibitor citalopram [6]. Thus, it is obvious that a transporter-mediated release potentiates the effects of a releasing drug. However, pure monoamine uptake inhibitors like cocaine [40], methylphenidate [4], or MDPV [41] exert considerable psychostimulant effects through transporter blockade alone, suggesting that release, in particular of DA, is not essential for psychostimulation. One could argue that the information about the releasing properties of a drug is non-relevant if the net neurotransmitter concentration in the synaptic cleft is considerably increased due to drug action at monoamine transporters. However, substrate-type psychostimulants affect also synaptic physiology intracellularly, which makes an essential difference to non-releasing drugs. Physiologically, monoaminergic neurotransmitters are, after their uptake into the presynapse, removed from the cytosol by uptake into storage vesicles via the vesicular monoamine transporter 2 (VMAT2). If anyhow remaining in the cytosol, they are rapidly metabolized via mitochondrial monoamine oxidases (MAO) or intracellular catechol-*O*-methyltransferase (COMT) [16]. Amphetamines disturb vesicular accumulation of neurotransmitters into vesicles [42] and also inhibit MAO [4]. The resulting increased cytosolic availability of neurotransmitters is potentially toxic for the neuronal cell [7]. This fact should be considered for the interpretation of pharmacological in vitro data of abused drugs, but in particular also of therapeutic drugs. For example, amphetamine and methylphenidate are both approved for treatment of attention deficit hyperactivity disorder, but since amphetamine is a substrate of DAT while methylphenidate is a pure uptake inhibitor, methylphenidate might be the gentler therapeutic drug [4].

4. Psychostimulant effects of amphetamines

In the following two sections, the psychostimulant or subjective effects of MDMA and methamphetamine will be described and explained by their mechanism of action. The description of these two prototype drugs, MDMA being rated as a predominantly entactogenic and methamphetamine as stimulant drug, would serve as model to predict expected effects in humans from in vitro results.

4.1. Subjective effects in humans induced by the entactogen MDMA

MDMA acts on SERT, NET, and DAT by inhibiting the reuptake of 5-HT, NE, and DA, respectively, but also by inducing transporter-mediated release of the respective monoamines. It acts thereby more potently on SERT and NET than on DAT [25]. MDMA inhibits the monoamines metabolizing enzyme MAO [43]. It also inhibits VMAT2 [44], preventing it to transport monoamines into storage vesicles. Both MDMA's effects on MAO and VMAT2 increase the cytosolic concentrations of monoamines.

MDMA's key psychostimulant effects are described as entactogenic, which means that the drug induces a subjective delightful psychotropic effect. This includes the feeling of closeness to others, relaxation, feeling of happiness and communicative openness [45]. MDMA lifts mood, increases empathy and confidence, promotes extroversion, increases self-esteem, and intensifies sensory perception. Psychomotor drive is only slightly increased [6].

Human studies with drugs of abuse are difficult to perform but are the only definite way to associate drug's subjective effects with the mode of action of a drug. Inhibition of SERT with the selective 5-HT reuptake inhibitor citalopram resulted in a reduction of most subjective MDMA effects. Euphoria, increased extroversion, and self-confidence were prominently reduced [6, 46]. This study documented that the psychological effects of MDMA are mostly due to 5-HT release [6]. In a similar study, pretreatment with the 5-HT_2 receptor antagonist ketanserin abolished the mild perceptual changes and emotional excitation induced by MDMA [46]. Another study showed the attenuation of the euphoriant amphetamine-like aspects of MDMA by pretreatment with the DA D_2-receptor antagonist haloperidol [46]. Liechti and Vollenweider concluded most subjective effects of MDMA to result from its effect at SERT (induction of 5-HT release and reuptake inhibition). Particular effects also

result from indirect action at specific monoamine postsynaptic receptors, since increased monoamine levels due to SERT and DAT modulation alter postsynaptic receptor stimulation [46]. Studies elucidating the role of NE in MDMA's mode of action are part of this thesis project.

4.2. Subjective effects of the stimulant methamphetamine

In humans, methamphetamine (30 mg) produces mostly positive mood effects, euphoria, excitation, reduced tiredness, cardiostimulant effects (e.g., increased heart rate, elevated blood pressure, pupil dilatation, and hyperthermia), reduced appetite, and erratic behavior [7]. Lasting for a short time period, cognitive performance including attention is improved. Users describe the performance enhancing effects of methamphetamine as increased energy and increased desire for work and resulting higher work performance [47]. Anxiety is a common negative effect of methamphetamine. All previously described effects seem to increase dose-dependently [7]. Doses higher than 30 mg could provoke psychotic symptoms. Also aggression, sustained talking, headaches, and hypertension are induced at high doses [48].

Methamphetamine releases DA, NE, and 5-HT in a transporter-mediated way, inhibiting all monoamine transporters for monoamine reuptake [49]. Thereby it acts more potently at DAT and NET than at SERT [5, 49]. Methamphetamine also acts on VMAT2, releasing stored monoamines from the vesicles into the cytosol and preventing uptake into vesicles via VMAT2. Inhibiting MAO [50], methamphetamine prevents monoamines from metabolism. Taking these mechanisms together, they all lead to an increase of monoamines in the synaptic cleft and thus to an enhanced neurotransmission, which is the basic concept to explain methamphetamines diverse effects [4]. In detail, excitation or arousal, one of the most prominent effects of methamphetamine, results from α_1- and β-adrenergic- and D_1- and D_2-receptor stimulation due to increased NE and DA concentrations in the synaptic cleft. Euphoria results from DA release in the nucleus accumbens. A generalized state of relaxation, in contrast, results from methamphetamines effects on serotonergic synapses. Improved cognitive performance mainly results from activation of the dopaminergic system, with α- and β-adrenergic receptors playing a role as well [7].

5. Adverse effects and complications of stimulant drug use

5.1. General overview on amphetamine-type drug-associated medical complications

Amphetamine-type drugs like MDMA and methamphetamine activate the sympathetic nervous system, from which many acute negative effects derive [7, 51]. The cardiostimulant effects include high blood pressure, increased heart rates, and hyperthermia. MDMA and methamphetamine typically also increase muscle tensions, resulting e.g., in bruxism, and induce sleep disturbances [51]. Psychiatric acute complications might be uncontrolled agitation and psychosis.

A typical methamphetamine-overdose includes agitation, pupil dilatation, tachycardia, hypertension and rapid respiration. Dangerous complications are hyperpyrexia and cardiac, hepatic, or renal failure [7]. Hyperthermia, probably resulting in hyperpyrexia, occurs frequently in hot environment like crowded clubs. Hyperthermia stimulates serious complications like rhabdomyolysis, myoglobinuria, acute renal failure, dissiminiated intravascular coagulation, cerebral hemorrhage, cardiac arrest, and liver failure [51, 52]. Apart of secondary effects of hyperthermia, hepatotoxicity and renal toxicity are important severe complications known to result from MDMA and methamphetamine ingestion, and hyponatremia as typical MDMA complication [7, 52].

5.2. Hyponatremia – a potentially life-threatening complication of MDMA

Hyponatremia derives from an inadequate secretion of antidiuretic hormone (syndrome of inappropriate antidiuretic hormone secretion (SIADH)) controlled by the hypothalamus, which results in increased renal water retention. SIADH is reflected by increased arginine vasopressin plasma levels [51] and results from the 5-HT releasing property of MDMA [53, 54]. Massive sweating and excessive liquid intake also trigger hyponatremia [52]. Hyponatremia can cause life-threatening brain edema with seizures and respiration or circulation failures [52]. Affected cases display bizarre behavior accompanied with vomiting, drowsiness, reduced consciousness or coma and probably seizures [55]. Interestingly, hyponatremia is a phenomenon observed in MDMA-taking women but rarely in men [53].

5.3. Neurotoxicity – a short disquisition

Speaking of adverse events and complications of MDMA and other amphetamine-type drugs, the question of their neurotoxic potential comes up. MDMA is believed to be a classical neurotoxic drug. Indeed, animal experimentations with amphetamines, performed intensively for years, showed a persistent reduction in brain levels of neurotransmitters, e.g., for MDMA mainly a reduction in 5-HT and for methamphetamine a reduction in DA levels [56, 57]. The enzymes involved in monoamine synthesis, tryptophan hydroxylase in case of 5-HT and tyrosine hydroxylase in case of DA and NE, are reduced due to drug abuse. Binding studies using positron emission tomography in vivo detected reduced levels of monoamine reuptake transporters in animals, but also in humans [7, 56, 58]. Cytotoxicity of the neuron itself is controversially discussed, since species differences and dose scaling result in an unclear picture [56]. Also, several studies observed that MDMA administered intra-cerebrally did not induce neurotoxicity [51]. Since MDMA, if not administered systemically but directly injected into the brain, evades its hepatic metabolism, one or more of MDMA's metabolites (see Figure 6) must be responsible for its toxicity [59].

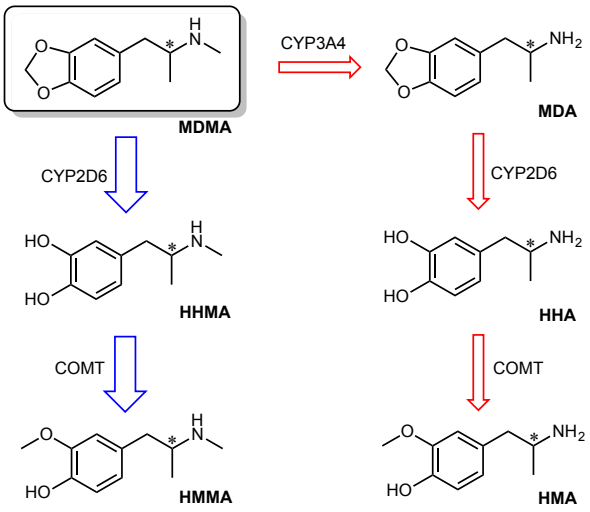

Figure 6: MDMA metabolism with the major pathway (blue) and the minor pathway (red). HHMA = 3,4-di-hydroxymethamphetamine; HMMA = 3-hydroxy-4-methoxymethamphetamine; MDA = 3,4-methylenedioxy-amphetamine; HHA = 3,4-dihydroxyamphetamine; HMA = 3-hydroxy-4-methoxyamphetamine. Pathway adapted from De La Torre et al. [60] as reviewed by Baumann et al. [56].

Concluding the findings of years of intense research on this topic, the term *neurotoxicity* should be reserved for compounds that damage nerve cells and be kept

apart from MDMA-associated neuronal changes including reduced neurotransmitter markers and down regulation mechanisms. There are hardly real structural damages with loss of neurons and resulting structural brain damage. How far neurotoxicity found in rodents is relevant for negative effects in humans is a matter of ongoing discussion. Assessment of neurotoxicity in humans is highly delicate since control groups usually do not perfectly match with the drug user group and there is confounding by life-style and other drugs used. Symptoms of depressed mood frequently observed in the days following MDMA use have also been linked to the sleep disruption associated with party drug use and clubbing rather than with the drug itself [61]. Often, people in the drug user group suffer from underlying psychological problems, take a variety of additional drugs, or they live a different daily routine than those in the control group [58]. One outstanding study matched the MDMA-taking group with the control group perfectly by nightlife habits (since sleep deprivation could bias MDMA related disorders, as the drug is mostly used at night-parties) and by the exclusion of other illicit drug use and massive alcohol consumption. Interestingly, this study failed to demonstrate cognitive impairment due to MDMA use [62].

5.4. Addiction – reward mechanisms

Dependence, an adapted state of neuronal cells or brain systems due to excessive drug use, can lead to addiction, the compulsive drug use despite negative physical, social, or economic problems [63]. Studies on cues that induce drug craving in addicted persons associate mainly the prefrontal cortical regions and the amygdalae, in some cases also the nucleus accumbens, with drug addiction. The dopaminergic system plays a key role in reward (situations rated by the brain as positive experiences) and reinforcement (urge to repeat behaviors that are associated with the respective drugs) of addictive drugs [63]. It projects from the midbrain ventral tegmental area to the nucleus accumbens, the dorsal striatum and other forebrain sites. Being a major neurotransmitter in the dopaminergic system, DA is the crucial neurotransmitter involved in the development of addiction. Generally spoken, addictive drugs pharmacologically act by increasing DA in the synaptic cleft of neurons in the nucleus accumbens [63, 64]. Altered DA release causes synaptic plasticity, as well as changes in neurotransmitter receptors or receptor-mediated signaling. Synaptic plasticity, the

ability of synapses to regulate their strength of neuronal transmission according to the neuronal activity, is a mechanistic cause of addiction [63].

Sulzer explains in his review in more details the crucial role of DA enhancement for the development of drug dependence [64]. DA enhancement in the synaptic cleft due to drug application can occur via four mechanisms:

1. The enhancement of neuronal firing (e.g., nicotine, opiates),
2. Inhibition of DA reuptake (cocaine as typical representative of this class),
3. Alteration of release probability from the presynapse (e.g., nicotine, opiates), and
4. DAT-mediated DA release (specialty of amphetamines).

Amphetamine-type drugs cause both DA uptake inhibition (second mechanism) and DA release (forth mechanism) and are anticipated to be rather addictive drugs. Serotonergic system activation, on the other hand, is understood to be protective against addiction [65]. For example, serotonergic neurons can control DA neurons and thus DA neurotransmission, via 5-HT_{2C} receptor activation [66, 67]. For a complete understanding of the addictive potential of a drug, it is necessary to assess both, effects on DA and 5-HT system activation [65, 68, 69].

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Objectives

Questions to be answered

For this thesis, we investigated different aspects of the mechanism of action of amphetamine-type designer drugs. All aspects were related to the drugs' pharmacology at monoamine transporters. We aimed to increase the understanding of the relation between in vitro pharmacology and human psychostimulant effects and drug-related complications such as acute or long-term medical problems. We focused our investigations on well-investigated substances like MDMA and methamphetamine as well as on hardly studied novel designer drugs. Taking MDMA as exemplary entactogenic drug, we aimed to assess which of the psychotropic and cardiostimulant effects in humans derive from transporter-mediated NE and 5-HT release. We also asked if cerebral NE/5-HT release blockade could prevent inappropriate secretion of antidiuretic hormone that frequently results in potentially life-threatening complications. With the stimulant and highly addictive methamphetamine, we chose an exemplary substance to work on pharmacological possibilities of addiction treatments. Thereby, we wanted to compare different therapeutics that target DAT. While these studies had a pronounced mechanistic focus, we also aimed to compile an extended set of descriptive in vitro experiments. We described novel but widely abused designer drugs and compared their pharmacological characteristics with well-investigated drugs, in particular with MDMA and methamphetamine. We aimed therewith to link descriptive in vitro pharmacology with a mechanistic understanding of positive or negative effects in humans. By improving the comprehension of the link between simple pharmacological in vitro tests and consequences of drug-use in humans, our research should provide a better understanding of designer drugs' pharmacology and toxicology and support medical treatment of acute and long-term drug use. In particular, we aimed to throw light on the actual effects of designer drugs such as MDPV, about which media reports terrify both general public and medical personnel.

Story line

Reading this thesis, the reader is first guided through the key pharmacological mechanism of amphetamine-type designer drugs in chapter 1 that contains two articles. One learns how drugs with similar chemical structures act differently on DAT, SERT, and NET in their potency and mechanism. One also learns to classify these designer drugs according to their pharmacological differences and which consequences on human effects can be drawn from in vitro pharmacological tests. In chapter 2, the reader will learn about the monoaminergic targets involved in the psychostimulant and cardiac effects of the entactogen MDMA, but also about the role of monoaminergic neurotransmitters in the development of hyponatremia, a potentially life-threatening complication of MDMA use. Finally, in chapter 3, the reader comes to know about key mechanisms in the development of stimulant addiction, which is a severe long-term complication for many drugs presented in chapter 1, and learns how these processes could pharmacologically be modulated. Presenting the research performed during my PhD studies in this composition, I aim to guide the reader through a comprehensive overview of amphetamine-type drugs' pharmacology and to point to its consequences either of less-addictive empathogen drugs like MDMA, or on highly-addictive stimulants like methamphetamine. By focusing strongly on mechanistic background of these observations, I also present pharmacological opportunities to find solutions against these complications.

Contributions

My main contribution to all publications and reports in this thesis was the establishment and in large part the accomplishment of all pharmacological in vitro assays. Exceptions were the in vitro blood-brain barrier tests and in vitro affinity assays, which were performed by collaborators. My contributions to the clinical studies were analytical measurements, namely the determination of plasma concentrations of MDMA, of metabolites, and of co-treatments. I also quantified copeptin and vasopressin in plasma samples. The analytical methods for the small molecules determinations were either developed and validated independently or in team-work. I am the first or co-equal first author on all papers and reports presented in this thesis, with the exception of paper 2a where I am the second author. This publication is included in my thesis since I made a significant effort for the analytical method development and for the measurement of analytes for pharmacokinetic assessments, and because the findings in paper 2a are valuable complementary information to paper 2b.

Chapter 1: Pharmacological description and classification of amphetamine-type designer drugs in vitro

This chapter contains two studies assessing the pharmacological profiles of amphetamine-type drugs and comparable stimulants in vitro. We tapped the full potential of classical pharmacological assays to determine potencies and the mode of action at a comprehensive set of monoaminergic pre- and postsynaptic targets. In paper 1a, we assessed the cathinones mephedrone, flephedrone, methylone, ethylone, butylone, cathinone, methcathinone, naphyrone, MDPV, pyrovalerone and compared their pharmacology with the non-β-keto amphetamines MDMA, 3,4-methylene-dioxyphenyl-N-methyl-2-butanamine (MBDB), 3,4-methylenedioxy-N-ethylamphe-tamine (MDEA), amphetamine, methamphetamine, and the non-amphetamine cocaine.

Paper 1b is a complementary study on the pharmacology of second series of cathinone-derivatives and non-β-keto analogs. We assessed the monoaminergic actions of the cathinones methedrone, 4-methylethcathinone (4-MEC), N,N-dimethylcathinone, ethcathinone, 3-fluoromethcathinone (3-FMC), buphedrone, pentylone, and pentedrone. We compared these cathinones to the non-β-keto amphetamines *para*-methoxymethamphetamine (PMMA), *para*-methoxy-amphetamine (PMA), 4-methylthioamphetamine (4-MTA), and N-ethylamphetamine.

Having performed identical pharmacological assays in both papers 1a and 1b, we are able to directly compare the pharmacology of all 28 substances at 15 monoaminergic targets. The size and integrity of our extensive designer drugs characterization is to date unique and gives a comprehensive overview of the pharmacology of designer drugs with original data.

Content of chapter 1:

Paper 1a

Pharmacological Characterization of Designer Cathinones In Vitro

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RESEARCH PAPER

Pharmacological characterization of designer cathinones *in vitro*

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BACKGROUND AND PURPOSE

Designer β-keto amphetamines (e.g. cathinones, 'bath salts' and 'research chemicals') have become popular recreational drugs, but their pharmacology is poorly characterized.

EXPERIMENTAL APPROACH

We determined the potencies of cathinones to inhibit DA, NA and 5-HT transport into transporter-transfected HEK 293 cells, DA and 5-HT efflux from monoamine-preloaded cells, and monoamine receptor binding affinity.

KEY RESULTS

Mephedrone, methylone, ethylone, butylone and naphyrone acted as non-selective monoamine uptake inhibitors, similar to cocaine. Mephedrone, methylone, ethylone and butylone also induced the release of 5-HT, similar to 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and other entactogens. Cathinone, methcathinone and flephedrone, similar to amphetamine and methamphetamine, acted as preferential DA and NA uptake inhibitors and induced the release of DA. Pyrovalerone and 3,4-methylenedioxypyrovalerone (MDPV) were highly potent and selective DA and NA transporter inhibitors but unlike amphetamines did not evoke the release of monoamines. The non-β-keto amphetamines are trace amine-associated receptor 1 ligands, whereas the cathinones are not. All the cathinones showed high blood–brain barrier permeability in an *in vitro* model; mephedrone and MDPV exhibited particularly high permeability.

CONCLUSIONS AND IMPLICATIONS

Cathinones have considerable pharmacological differences that form the basis of their suggested classification into three groups. The predominant action of all cathinones on the DA transporter is probably associated with a considerable risk of addiction.

Abbreviations

BBB, blood–brain barrier; DA, dopamine; DAT, dopamine transporter; MBDB, 3,4-methlyenedioxyphenyl-N-methyl-2-butanamine; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDPV, 3,4-methylenedioxypyrovalerone; NET, NA transporter; P_e, permeability coefficient; SERT, 5-HT transporter; TA receptor, trace amine-associated receptor

Introduction

Stimulant drug abuse remains a major public health issue worldwide. While 'old stimulants', including cocaine, methamphetamine and amphetamine, and 'entactogens', including 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), 3,4-methylenedioxy-N-ethylamphetamine (MDEA) and 3,4-methlyenedioxyphenyl-N-methyl-2-butanamine

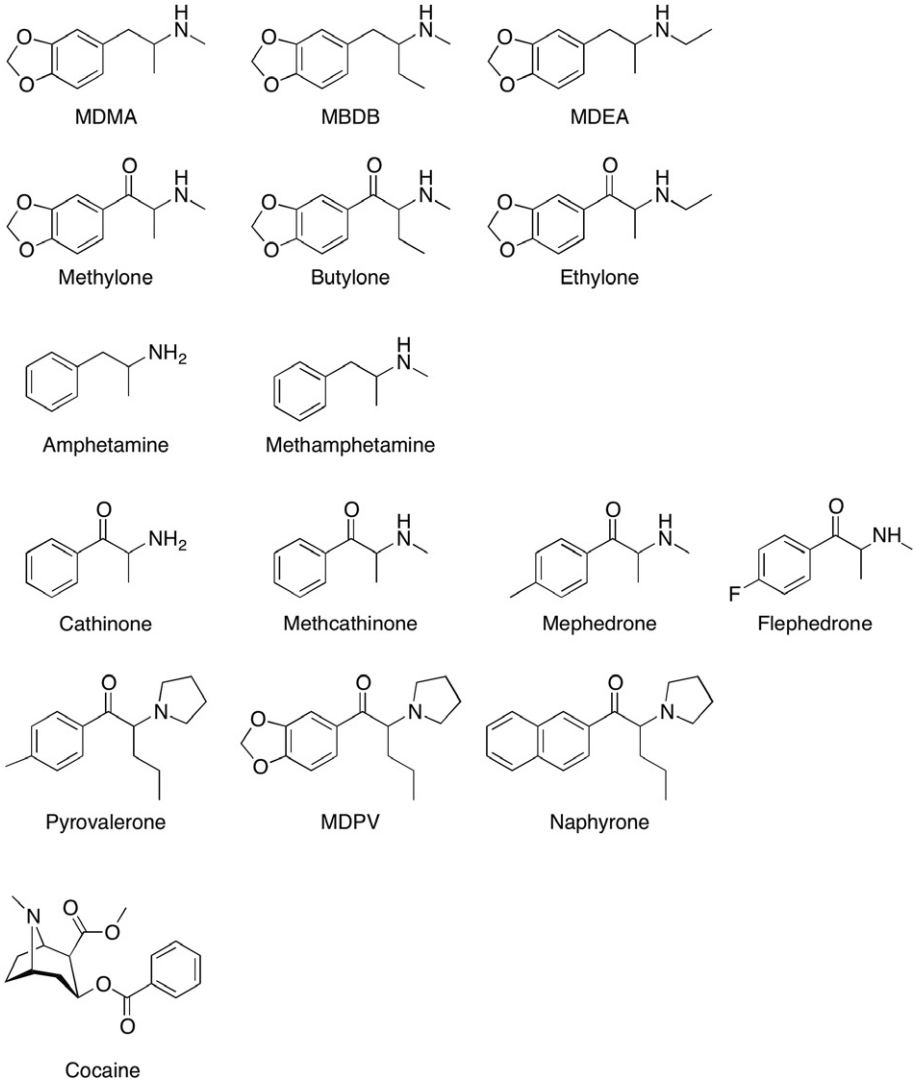

Figure 1

Chemical structures of cathinones, related amphetamines and cocaine.

(MBDB), continue to be used, novel designer cathinones are emerging. Cathinones differ from amphetamines by the presence of a ketone oxygen group at the β-position (Figure 1). The β-keto-amphetamines are distributed as 'bath salts', 'research chemicals' and 'plant food' via the Internet and have been advertised as 'legal highs' with similar psychotropic effects to MDMA or cocaine (Spiller *et al.*, 2011).

As β-keto analogues of amphetamines, cathinones may be expected to have amphetamine-like effects because of their structural similarity. Cathinones enhance DA, NA and 5-HT neurotransmission (Hadlock *et al.*, 2011; Kehr *et al.*, 2011; Baumann *et al.*, 2012; Lopez-Arnau *et al.*, 2012; Martinez-Clemente *et al.*, 2012). However, the molecular pharmacology of this novel class of stimulant drugs is poorly documented. In particular, a systematic comparative characterization of the effects of different cathinones on the human DA, NA and 5-HT transporters and comparisons with classic stimulants are lacking.

In the present study, we assessed the *in vitro* pharmacology of cathinone, methcathinone, mephedrone (4-methylmethcathinone), flephedrone (4-flouromethcathinone), methylone (3,4-methylenedioxymethcathinone, β-keto-MDMA), ethylone (3,4-methylenedioxyethylcathinone, β-keto-MDEA), butylone (β-keto-MBDB), pyrovalerone, 3,4-methylenedioxypyrovalerone (MDPV) and naphyrone (naphthylpyrovalerone). We determined the potencies of these cathinones to inhibit DA, NA and 5-HT transport *in vitro*. We also tested whether cathinones are releasers of DA or 5-HT and characterized the binding affinities of these drugs for monoamine transporters, dopamine D_{1-3} receptors, α_1 and α_2 adrenoceptors, 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, the trace amine-associated receptor 1 (TA_1 receptor) and the histamine H_1 receptor. Finally, blood-brain–barrier (BBB) permeability was assessed using a human *in vitro* model. The pharmacological profiles of the novel cathinones were compared with their non-β-keto amphetamine analogues,

including MDMA, MDEA, MBDB, amphetamine and methamphetamine as well as with cocaine.

Methods

The drug target nomenclature conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Drugs

The hydrochloride salts of the drugs (purity >98.5%) were supplied by Lipomed (Arlesheim, Switzerland), with the exception of naphyrone, which was synthesized according to Meltzer *et al.* (2006). Racemic drugs were used except for D-amphetamine and D-methamphetamine.

Radioligand binding

The radioligand binding assays were performed as described previously (Revel *et al.*, 2011; Hysek *et al.*, 2012c). Briefly, membrane preparations from HEK 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective human transporters (Tatsumi *et al.*, 1997) or receptors (except for rat/mouse TA_1 receptor) (Revel *et al.*, 2011) were incubated with the radiolabelled selective ligands at concentrations equal to K_d, and ligands 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 were used: *N*-methyl-[^{3}H]-nisoxetine and indatraline (NA transporter [NET]), [^{3}H]-citalopram and indatraline (5-HT transporter [SERT]) and [^{3}H]-WIN35,428 and indatraline (DA transporter [DAT]). [^{3}H]-8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) and indatraline ($5\text{-}HT_{1A}$ receptor), [^{3}H]-ketanserin and spiperone ($5\text{-}HT_{2A}$ receptor), [^{3}H]-mesulergine and mianserin ($5\text{-}HT_{2C}$ receptor), [^{3}H]-prazosin and risperidone (α_1 adrenoceptor), [^{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 (histamine H_1 receptor) and [^{3}H]-RO5166017 and RO5166017 (TA_1 receptor). All radioligands were obtained from Perkin-Elmer (Schwerzenbach, Switzerland), with the exception of [^{3}H]-RO5166017, which was synthesized at Roche (Basel, Switzerland).

Monoamine uptake transporter inhibition

The potencies of the drugs to inhibit the SERT, NET and DAT were evaluated in HEK 293 cells that stably expressed human SERT, NET and DAT (Tatsumi *et al.*, 1997) as previously described (Hysek *et al.*, 2012c). The DAT/SERT ratio was calculated as 1/DAT IC_{50}:1/SERT IC_{50}.

Monoamine release

We assessed DAT- and SERT-mediated DA and 5-HT efflux in HEK 293 cells that overexpressed human DAT or SERT respectively. We cultured the cells in 24-well plates (XF24, Seahorse Biosciences, North Billerica, MA) coated with poly-D-lysine to 70–100% confluency. After removing the culture medium, we added 85 µL release buffer (Krebs–HEPES that contained 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 10 mM D-glucose, 0.2 mg·mL^{-1} ascorbic acid and 10 µM pargyline) with 10 nM [^{3}H]-5-HT (SERT cells) or 10 nM [^{3}H]-DA and 1 µM unlabelled DA (DAT cells). We filled the cells with the respective radiolabelled monoamine for 20 min at 37°C. We then removed the buffer and washed twice with warm buffer. We induced [^{3}H]-5-HT and [^{3}H]-DA release by adding 1000 µL release buffer that contained the drugs in different concentrations or controls. We incubated the cells for 15 min at 37°C and shaked at 300 r.p.m. on a rotary shaker. We then stopped the release by removing the buffer and washing the cells twice with ice-cold buffer. The release time was based on the difference between drug-stimulated and spontaneous release (control) over time, which reached its maximum before 15 min. We then added 65 µL lysis buffer and lysed the cells overnight in a refrigerator. We mixed 50 µL of the cell lysate suspension with 2.5 mL UltimaGold and determined radioactivity. The radioactive counts in the cells where no drug was present in the release buffer (control) was defined as 100%, and the percentages of radioactivity that remained in the cells treated with drugs were calculated. Pure uptake transporter inhibitors, including imipramine, citalopram, cocaine and mazindol, have been shown to produce apparent substrate efflux from monoamine-preloaded HEK cells that is explained by inhibition of transporter-mediated re-uptake of the substrate that diffuses out of the cells (Scholze *et al.*, 2000). The DAT inhibitor mazindol and SERT inhibitor citalopram reduced the amount of preloaded DA and 5-HT (mean ± SD) by 15.6 ± 7 and 19.6 ± 8%, respectively, at the maximal concentration of 100 µM (E_{max}) compared with controls. This nonspecific release was subtracted from total release at the maximal drug concentration of 100 µM to yield E_{max} values of specific transporter-mediated release. We considered any drug that produced significantly higher maximal DA efflux compared with mazindol to be a DA releaser and a drug that produced significantly higher maximal 5-HT efflux compared with citalopram as a 5-HT releaser. EC_{50} values were calculated using Prism (GraphPad, San Diego, CA). ANOVA followed by Dunnett's tests were used to compare drug effects with the control condition. Efflux was studied in DAT- and SERT-expressing cells because the action of a drug on the DA and 5-HT system was considered to be relevant for predicting its stimulant-like properties and abuse potential (Rothman and Baumann, 2006).

Cytotoxicity

Cell membrane integrity was verified using the ToxiLight BioAssay Kit (Lonza, Basel, Switzerland) for all of the drugs (10 and 100 µM) after 4 h of incubation at 37°C.

Transendothelial BBB transport

Transendothelial transport was assessed for a selection of compounds using a human *in vitro* BBB permeability model (Sano *et al.*, 2010; 2012). Conditionally immortalized human brain capillary endothelial cells (TY09) were obtained from the Department of Neurology and Clinical Neuroscience, Yamaguchi University, Japan. TY09 cells express the human blood-to-brain influx and brain-to-blood efflux transporters,

form tight cell monolayers and retain BBB-specific properties independent of cell passage number (Sano *et al.*, 2012). The cells were grown in growth medium (EGM-MV BulletKit CC-3125, Lonza, Verviers, Belgium) supplemented with 20% FBS (AMIMED, BioConcept, Allschwil, Switzerland), 100 U·mL^{-1} penicillin (Sigma, Buchs, Switzerland) and 100 mL·mL^{-1} streptomycin (Sigma). The cells were seeded on Transwell polycarbonate membrane inserts (Corning, Baar, Switzerland; 0.4 µm pore size, 12 mm insert diameter) precoated with rat tail collagen type 1 solution (Becton Dickinson, Allschwil, Switzerland) at a density of 5×10^4 cells·cm^{-2} and grown to confluence. Before the initiation of the transport studies, the cell culture medium was replaced with prewarmed transport buffer (HBSS supplemented with 10 mM HEPES and 1 mM Na-Pyruvate, pH 7.4), and 1.5 µM of the test substance was added to the donor compartment of a Transwell filter insert. The extracellular marker Lucifer yellow CH dilithium salt (Sigma) was always combined with the test compound in the same experiment to provide a control for cellular tightness. The initial concentration of Lucifer yellow applied was 10 µM. After 10, 20, 30, 45 and 60 min, 200 µL samples were collected from the acceptor compartment and replaced by buffer. Additionally, a sample of 200 µL was taken from the stock solution and analysed. Lucifer yellow was quantified by fluorescence spectroscopy using a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Drug concentrations were determined using HPLC coupled to tandem MS. The analytes were extracted using methanol that contained 0.1 µg·mL^{-1} MDMA-d5 (Lipomed, Arlesheim, Switzerland). Chromatographic separation was performed on a Shimadzu HPLC system (Shimadzu, Reinach, Switzerland). A Reprosil Fluosil 100 PFP column (50 × 2 mm, 2.2 µm, Dr Maisch, Ammerbuch-Entringen, Germany) was used for the separation of the analytes. Eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in methanol) were used with the following gradient: 5% B for 0–0.4 min, 5–98% B for 0.4–1.9 min, 98% B for 1.9–2.7 min, and 5% B for 2.7–3 min. The mobile phases were delivered at a constant flow rate of 0.35 mL·min^{-1}. The total run time was 3.0 min. The column oven was set at 40°C. The injection volume was 10 µL. MS detection was performed using a triple quadrupole MS (API3200, Applied Biosystems, Rotkreuz, Switzerland) operated in electrospray ionization positive-ion mode. The assays were linear in the concentration range of 1.2–300 ng·mL^{-1} for all of the analytes. The selected mass-to-charge (*m/z*) ratio transitions of the protonated MH$^+$ analyte ions used in selective reaction monitoring mode were the following: MDMA 194 → 163, MDMA-d5 199 → 165, mephedrone 178 → 160, methylone 208 → 160, cocaine 304 → 182, cathinone 150 → 132, methcathinone 164 → 146, amphetamine 136 → 91, methamphetamine 150 → 91, MDPV 276 → 126. The dwell time was set at 20 ms for all of the analytes.

Permeability coefficients were calculated according to Equations 1–3 (Cecchelli *et al.*, 1999):

$$\text{Clearance (mL)} = \text{Cl}^- \text{ (mL)} = X/C_d \quad (1)$$

where X is the cumulative amount of drug transported to the acceptor compartment, and C_d is the concentration of the substance in the donor compartment at each time point. C_d is calculated by subtracting the accumulated transported amount of drug from the initial amount in the donor compartment determined from the stock solution. *Cl* refers to the total cleared volume at each time point. The permeability-surface area product (*PS*) is determined by plotting *Cl* as a function of time. The slope of the curve represents the *PS* value. The *PS* values of the cell monolayer plus filter (PS_{total}) and porous filter (PS_{filter}) were determined and used for the calculation of the permeability coefficient (P_e) according to the following equations:

$$1/PS_e = 1/PS_{total} - 1/PS_{filter} \quad (2)$$

$$P_e = PS_e/A \quad (3)$$

where A is the surface area of the filter. The P_e ratios were obtained by normalizing the P_e values of the test compounds (P_e *test*) with the corresponding P_e values of the extracellular marker Lucifer yellow (P_e *Lucifer yellow*): P_e *ratio* = P_e *test*/P_e *Lucifer yellow*. $P_e \leq 1$ indicates low trancellular permeability as observed with highly hydrophilic compounds, such as sucrose. $P_e > 1$ and <3 indicates intermediate permeability, and $P_e \geq 3$ indicates high permeability (Sano *et al.*, 2012). Estimates of partition coefficient (CLogP) values (Ghose *et al.*, 1998) were calculated using ChemDraw Ultra 11 (CambridgeSoft, Cambridge, MA).

Results

Receptor binding profiles

The monoamine transporter and receptor binding affinities are shown in Table 1. Pyrovalerone and MDPV exhibited very high affinity for the DAT and NET in the low nanomolar range (<10 nM), consistent with their high potency as DAT and NET inhibitors (Table 2). Cathinone and methcathinone showed similar monoamine transporter binding profiles to amphetamine and methamphetamine, with binding affinities for the DAT and NET in the low micromolar range (<10 µM) and no affinity for the SERT (>30 µM). Transporter binding affinities for the DAT and SERT were generally lower than the respective potencies as transporter inhibitors for those compounds that also released DA or 5-HT respectively. Mephedrone, flephedrone and methcathinone were the only cathinones that exhibited relevant (<10 µM) 5-HT$_{2A}$ receptor binding. These compounds and cathinone also bound to α_1 adrenoceptors, which was not seen for the other drugs investigated. Cocaine and all of the cathinones showed lower binding affinity for TA$_1$ receptor compared with the non-β-keto analogue amphetamines.

Inhibition of monoamine transporters

The effects of the cathinones and reference substances on monoamine transporter function are shown in Figure 2. IC$_{50}$ values for monoamine transport inhibition and DAT/SERT inhibition ratios are shown in Table 2. Significant differences were observed in the absolute and relative potencies of the cathinones to inhibit monoamine transporter function. Pyrovalerone and its derivative MDPV were the most potent DAT inhibitors, significantly more potent than all of the

Table 1
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	TA$_{rat}$	TA$_{mouse}$
MDMA	30.5 ± 8.0	6.5 ± 2.5	13.3 ± 0.6	12.2 ± 0.8	7.8 ± 2.4	>13	>6	15.0 ± 10	>13.6	25.2 ± 12	>17.7	>14.4	0.37 ± 0.12	2.4 ± 1.1
MBDB	11.9 ± 1.4	3.1 ± 0.3	5.80 ± 0.7	15.5 ± 3.8	6.62 ± 1.8	>13	>6	16.9 ± 4	>12.5	NA	>16	>13	1.2 ± 0.1	3.6 ± 1.1
MDEA	6.17 ± 1.4	1.35 ± 0.06	4.32 ± 0.6	18.8 ± 0.78	>13	>13	>6	12.2 ± 0.5	>12.5	NA	>16	>13	0.32 ± 0.6	4.9 ± 1.7
Ethylone	9.89 ± 0.9	1.43 ± 0.4	9.04 ± 0.6	17.0 ± 2.4	>13	>13	>6	>25	>12.5	NA	>16	>13	>12.5	>10
Mephedrone	>25	3.4 ± 0.8	>30	>20	2.1 ± 0.7	>13	3.48 ± 2.2	11.0 ± 5.0	>13.6	>30	>9.2	>14.4	4.3 ± 2.0	>10
Naphyrone	0.18 ± 0.02	0.04 ± 0.01	0.18 ± 0.02	6.00 ± 0.21	11 ± 2.2	>13	>6	8.0 ± 2.8	>12.5	NA	>16	2.28 ± 0.27	>12.5	>10
Butylone	8.13 ± 0.7	0.44 ± 0.03	14.1 ± 4.1	>20	>13	>13	>6	>25	>12.5	NA	>16	>13	>12.5	>10
Cocaine	4.47 ± 2.6	0.28 ± 0.07	1.1 ± 0.09	>20	>13	>13	>6	>20	>13.6	>30	>17.7	>14.4	>10	>10
Methylone	>25	2.73 ± 0.2	>30	>20	>13	>13	>6	>20	>13.6	>30	>9.2	>14.4	>12.5	>10
Flephedrone	>25	12.2 ± 3.1	>30	>20	1.4 ± 0.6	>13	1.52 ± 0.05	>20	>13.6	>30	>17.7	>14.4	5.4 ± 1.7	>10
Cathinone	3.50 ± 2.7	19.8 ± 1.9	>30	>20	>13	>13	5.40 ± 1.1	8.9 ± 2.7	>13.6	>30	>17.7	>14.4	2.2 ± 0.70	2.1 ± 0.73
Methcathinone	1.45 ± 0.7	1.28 ± 0.2	>30	12.7 ± 3.5	3.0 ± 0.6	>13	3.93 ± 1.3	11.9 ± 3.9	>13.6	>30	>9.2	>14.4	4.1 ± 1.2	>10
Amphetamine	1.00 ± 0.6	5.68 ± 3.8	>25	6.74 ± 1.38	>13	>13	>6	2.8 ± 0.8	>13.6	>30	>17.7	>14.4	0.23 ± 0.18	0.09 ± 0.06
Methamphetamine	4.28 ± 2.1	1.85 ± 0.9	26.7 ± 11	8.07 ± 0.75	>13	>13	>6	6.1 ± 1.6	>13.6	>30	>17.7	>14.4	0.35 ± 0.12	0.55 ± 0.2
Pyrovalerone	0.06 ± 0.005	0.03 ± 0.005	4.97 ± 0.3	13.4 ± 2.1	>13	>13	>6	>20	>13.6	>30	>9.2	10.7 ± 1.5	>12.5	>10
MDPV	0.08 ± 0.02	0.01 ± 0.002	2.86 ± 0.1	10.29 ± 4.7	>13	>13	>6	>20	>13.6	>30	>9.2	>14.4	7.2 ± 1.1	>10

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

other drugs. The rank order of potency for DAT inhibition was MDPV and pyrovalerone >> naphyrone, cocaine, methamphetamine, amphetamine and methcathinone > butylone, mephedrone, methylone, ethylone, flephedrone and MDEA > cathinone, MDMA and MBDB. The rank order of potency for SERT inhibition was naphyrone, MDEA and MDMA > MBDB, cocaine, ethylone, mephedrone and butylone >> all of the others. The DAT/SERT inhibition ratios ranged from >100 for pyrovalerone and MDPV (mostly DAT inhibition) to 0.08 for MDMA (mostly SERT inhibition). The entactogens MDMA, MBDB and MDEA were the only drugs that blocked the SERT significantly more potently than the DAT (DAT/SERT ratio << 1). Ethylone, mephedrone, naphyrone, butylone and methylone were similar to cocaine, with DAT/SERT selectivity ratios in the range of 1–4. Cathinone and methcathinone were similar to their non-β-keto analogues amphetamine and methamphetamine, with DAT/SERT inhibition ratios >10. The rank order of potency for NET inhibition was pyrovalerone and MDPV > methamphetamine, methcathinone and amphetamine > cathinone, flephedrone, naphyrone and mephedrone > MDMA, cocaine and methylone > MDEA, butylone, ethylone and MBDB. DAT and NET but not SERT inhibition potency (IC_{50}) values were correlated with psychotropic effective doses (Table 2) as reported from experimental studies (Martin *et al.*, 1971) or by recreational users (Derungs *et al.*, 2011) http://www.erowid.org; accessed June 20, 2012). The Spearman rank correlation coefficients were $r_s = 0.73$ and 0.79 respectively (both $P < 0.01$).

Monoamine release

Amphetamine, methamphetamine, cathinone, methcathinone, flephedrone, mephedrone and MDMA released DA through the DAT (Figure 3 and Table 3). However, the potency of MDMA to release DA was low (EC_{50} > 10 µM). The entactogens MDMA, MDEA and MBDB, as well as the cathinones methylone, ethylone, butylone and mephedrone released 5-HT through the SERT. Amphetamine, methamphetamine, methcathinone and flephedrone also released 5-HT, however, only at very high concentrations (EC_{50} > 33 µM). The pyrovalerone derivatives, including pyrovalerone, naphyrone and MDPV, produced no DA or 5-HT efflux similar to cocaine, indicating that these pyrovalerone derivatives act as very potent transporter inhibitors but not substrate releasers.

Cytotoxicity

None of the drugs showed apparent cytotoxicity at the concentrations used in the functional assays.

Transendothelial transport

All of the positively tested drugs exhibited P_e ratios ≥3, indicating high permeability (Table 4). P_e ratios for mephedrone and MDPV were >10, suggesting very high permeability. Additionally, the apical to basolateral transport of MDPV was significantly greater ($P < 0.05$) than basolateral to apical transport, consistent with active transport by one of the blood-to-brain influx carriers. P_e ratios could not be calculated for cocaine and cathinone because of low recovery.

Table 2

Monoamine transport inhibition

	NET IC_{50} (μM) (95% CI)	DAT IC_{50} (μM) (95% CI)	SERT IC_{50} (μM) (95% CI)	DAT/SERT ratio Ratio (95% CI)	Recreational dose* mg
MDMA	0.447 (0.33–0.60)	17 (12–24)	1.36 (1.0–2.0)	0.08 (0.04–0.16)	100
MBDB	2.80 (1.9–4.1)	22 (20–26)	2.04 (1.4–3.0)	0.09 (0.05–0.15)	200
MDEA	1.02 (0.78–1.3)	9.3 (8.0–11)	1.27 (0.93–1.7)	0.14 (0.01–0.21)	125
Ethylone	2.54 (2.0–3.2)	5.68 (4.9–6.5)	4.46 (3.8–5.2)	0.8 (0.6–1.1)	175
Mephedrone	0.254 (0.22–0.30)	3.31 (2.6–4.2)	4.64 (3.7–5.9)	1.4 (0.9–2.4)	150
Naphyrone	0.25 (0.20–0.32)	0.47 (0.40–0.55)	0.96 (0.85–1.09)	2.0 (1.5–2.7)	25
Butylone	2.02 (1.5–2.7)	2.90 (2.5–3.4)	6.22 (4.3–9.0)	2.1 (1.3–3.6)	150
Cocaine	0.451 (0.38–0.59)	0.768 (0.6–1.0)	2.37 (2.0–2.9)	3.1 (2–4.8)	75
Methylone	0.542 (0.39–0.75)	4.82 (3.8–6.1)	15.5 (10–26)	3.3 (1.5–6.8)	150
Flephedrone	0.246 (0.16–0.37)	6.35 (4.2–9.5)	>10	5.8 (0.8–41)	200
Cathinone	0.199 (0.15–0.26)	14.0 (10–20)	>100	>10	50
Methcathinone	0.085 (0.06–0.17)	1.12 (0.83–1.5)	>10	>10	59
Amphetamine	0.094 (0.06–0.14)	1.30 (0.83–2.0)	>10	>10	30
Methamphetamine	0.064 (0.04–0.09)	1.05 (0.74–1.5)	>10	>10	30
Pyrovalerone	0.043 (0.03–0.06)	0.035 (0.03–0.04)	13.0 (10.8–15.8)	>100	20
MDPV	0.044 (0.03–0.07)	0.031 (0.03–0.04)	9.30 (6.8–12.8)	>100	5

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

Discussion

All of the cathinones were inhibitors of the monoamine transporters, but their selectivity for the SERT, NET and DAT varied considerably. Further, most of the compounds were substrate releasers. Thus, important pharmacological differences were found between different cathinones. We classified the cathinones into three groups based on, firstly, their relative potency to act as SERT, NET and DAT inhibitors and, secondly, their action as substrate releasers: (1) cocaine-MDMA-mixed cathinones (including mephedrone, methylone, ethylone, butylone and naphyrone, which act as relatively nonselective monoamine uptake inhibitors similar to cocaine and, with the exception of naphyrone, also as MDMA-like 5-HT releasers); (2) methamphetamine-like cathinones (including cathinone, methcathinone and flephedrone, which act as preferential catecholamine inhibitors and DA releasers, similar to amphetamine and methamphetamine); and (3) pyrovalerone–cathinones (including pyrovalerone and MDPV, which act as very potent and selective catecholamine uptake blockers but not substrate releasers).

The potency of drugs of abuse to inhibit the NET and DAT or activate the NA and DA system is associated with their psychostimulant effects and enhanced abuse liability (Rothman *et al.*, 2001). Consistently, we found that the doses of the drugs abused by humans correlated with their potency to inhibit catecholamine transport. In contrast, relatively increased activation of the 5-HT system is linked to a reduction in abuse potential (Wee *et al.*, 2005; Rothman and

Table 3

Monoamine release from monoamine-preloaded cells

	DAT EC_{50} (μM) (95% CI)	SERT EC_{50} (μM) (95% CI)
MDMA	22 (8.9–53)	5.63 (3.5–9.2)
MBDB	>100	2.49 (1.0–6.9)
MDEA	>100	2.88 (1.6–5.0)
Ethylone	>100	9.90 (2.4–40)
Mephedrone	3.75 (1.7–8.4)	5.98 (3.2–11)
Naphyrone	>100	>100
Butylone	>100	5.5 (1.8–17)
Cocaine	>100	>100
Methylone	>100	>10
Flephedrone	12.5 (5.7–28)	>33
Cathinone	5.64 (3.0–10)	>100
Methcathinone	2.36 (1.7–3.3)	>33
Amphetamine	1.76 (1.1–2.9)	>33
Methamphetamine	1.56 (0.9–2.8)	>33
Pyrovalerone	>100	>100
MDPV	>100	>100

Values are means.

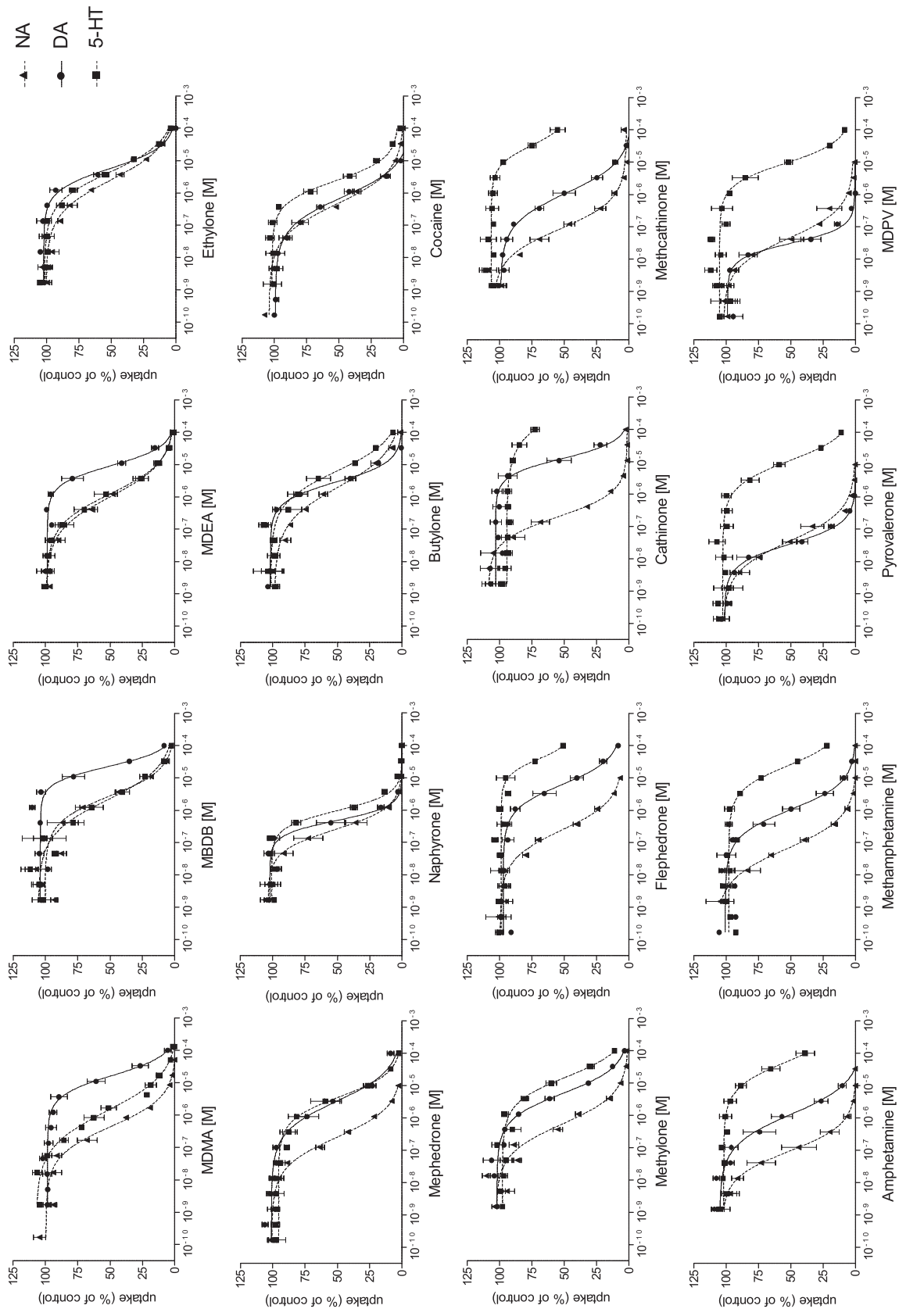

Figure 2
Monoamine uptake inhibition. Potencies of different drug concentrations to inhibit the accumulation of NA, DA and 5-HT into NA, DA and 5-HT transporter-transfected HEK 293 cells respectively. The data are expressed as the mean ± SEM of three to five independent experiments. The lines show the data fit by nonlinear regression. IC_{50} values are presented in Table 2.

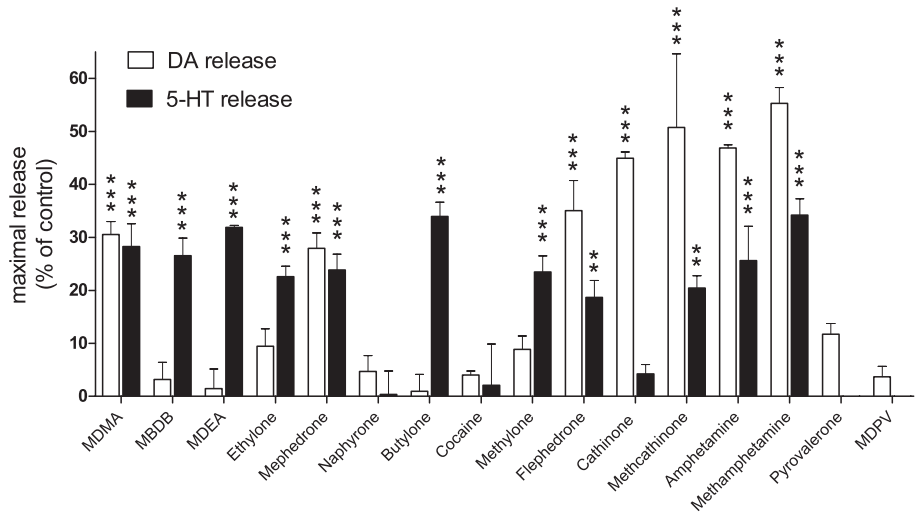

Figure 3

Dopamine and 5-HT release. HEK 293 cells that stably express the DA or 5-HT transporter were preloaded with [^{3}H]-DA or [^{3}H]-5-HT, respectively, washed and incubated with drugs. Transporter-mediated release is expressed as % reduction in monoamine cell content at the maximal drug concentration (100 μM) compared with controls. $^{**}P < 0.01$, $^{***}P < 0.001$, significant effects compared with controls. The EC_{50} values are shown in Table 3. The data are expressed as the mean ± SEM of three independent experiments.

Table 4

Blood-brain barrier permeability

	P_e ratio				
	Apical to basolateral	Basolateral to apical	Permeability	[a]Active transport	[b]CLogP
MDMA	6.0 ± 0.56	7.4 ± 2.4	+	No	1.85
Mephedrone	14.0 ± 10.4	12.2 ± 6.1	++	No	1.67
Methylone	6.1 ± 2.8	5.3 ± 1.3	+	No	1.39
Methcathinone	5.9 ± 2.8	8.5 ± 3.2	+	No	1.19
Amphetamine	6.3 ± 3.7	5.2 ± 1.3	+	No	1.74
Methamphetamine	5.4 ± 1.1	6.4 ± 3.0	+	No	1.74
MDPV	37.2 ± 11.3	12.0 ± 11.2	++	Yes	3.80

Data are expressed as mean ± SD (n = 3–9).
P_e ratios indicate the blood–brain barrier permeability of the drug in relation to the extracellular marker lucifer yellow (P_e = 1).
+, high permeability (P_e ratio >3). ++, very high permeability (P_e ratio >10).
[a]P <0.05 significant difference between apical to basolateral compared with basolateral to apical transport indicating active transport.
[b]CLogP, prediction of partition coefficient (lipophilicity).

Baumann, 2006; Baumann *et al.*, 2011) and more 'entactogenic' MDMA-like subjective drug effects (Liechti *et al.*, 2000a). Thus, the relative *in vitro* effect on the DAT versus SERT is useful to predict drug characteristics *in vivo* and compare novel cathinones with known psychostimulants. MDMA is selective for the SERT, with a DAT/SERT inhibition ratio of 0.08 (present study) and DA/5-HT release potency ratio <1 (Baumann *et al.*, 2012), and produces positive mood effects in humans with little psychostimulation (Liechti *et al.*, 2001). Cocaine has a DAT/SERT ratio close to unity, and methamphetamine is more selective for the DAT, with a DAT/SERT inhibition ratio >10 and DA/5-HT release ratio >100 (Baumann *et al.*, 2012) and has mostly psychostimulant effects in humans.

Cocaine-MDMA-mixed cathinones

Mephedrone, methylone, ethylone, butylone and naphyrone exhibited relative DAT versus SERT inhibition potencies in the range of 1–5, similar to cocaine. Uptake inhibition studies using rat synaptosomes found that mephedrone was equally potent at the DAT and SERT (Hadlock *et al.*, 2011). Methylone and butylone were slightly more potent DAT than SERT inhibitors at the human transporter as previously shown for methylone (Cozzi *et al.*, 1999). Equal uptake inhibition

potencies for the DAT and SERT were shown for methylone and butylone using rat brain synaptosomes (Nagai *et al.*, 2007; Lopez-Arnau *et al.*, 2012). Ethylone was an equipotent inhibitor of all three transporters, and we are not aware of other published data. Compared with methylone, ethylone and butylone, the respective non-β-keto analogue entactogens MDMA, MDEA and MBDB were 10-fold more selective for the SERT versus DAT, consistent with previous data on methylone and MDMA (Cozzi *et al.*, 1999; Nagai *et al.*, 2007). Together the data indicate that the cocaine–MDMA–mixed cathinones are more dopaminergic with regard to monoamine transporter inhibition than their entactogen analogs.

In terms of monoamine release, the cocaine–MDMA–mixed cathinones were comparable with MDMA. Ethylone and butylone released 5-HT, comparable with their non-β-keto entactogen analogues MBDB and MDEA, but with lower potency. Previous studies found that the monoamine release profiles of mephedrone and methylone resembled those of MDMA, with DAT/SERT and NET/DAT ratios close to unity (Nagai *et al.*, 2007; Baumann *et al.*, 2012). However, mephedrone was a more potent releaser of DA than MDMA in the present study and from striatal suspensions preloaded with DA (Hadlock *et al.*, 2011). An *in vivo* microdialysis study in rats showed that mephedrone also produced a rapid and pronounced increase in nucleus accumbens DA levels, comparable with amphetamine and unlike MDMA, which only moderately elevates DA levels (Kehr *et al.*, 2011). Both mephedrone and MDMA also produced strong increases in extracellular 5-HT, whereas amphetamine had only a moderate effect on 5-HT levels (Kehr *et al.*, 2011). Other microdialysis studies showed that mephedrone and methylone elevated extracellular DA and 5-HT levels in the rat nucleus accumbens, with relatively higher effects on 5-HT levels (Aarde *et al.*, 2011; Baumann *et al.*, 2012), similar to MDMA and unlike methamphetamine, which preferentially increases DA (Baumann *et al.*, 2012). Thus, mephedrone shares some of the DA-releasing properties of amphetamine and methamphetamine and 5-HT-releasing property of MDMA. Mephedrone also produced relatively weak motor stimulation similar to MDMA, unlike amphetamine that strongly increases locomotor activity in rats (Kehr *et al.*, 2011), and a preference to move along the walls of the test box (Motbey *et al.*, 2012) as previously described for MDMA. Like MDMA, mephedrone also reduced voluntary wheel running in rats, while running was increased by methamphetamine or MDPV (Huang *et al.*, 2012). Similar to mephedrone, methylone was also reported to be a weak motor stimulant compared with methamphetamine (Baumann *et al.*, 2012). Drug discrimination studies in rats also showed that methylone generalized well to MDMA and at lower potency also to amphetamine (Dal Cason *et al.*, 1997). Mephedrone is self-administered by rats (Aarde *et al.*, 2011; Hadlock *et al.*, 2011), has been reported to produce strong craving in humans (Brunt *et al.*, 2011) and when administered intranasally is rated by users to be more addictive than cocaine (Winstock *et al.*, 2011). Furthermore, mephedrone showed very high BBB permeability in our *in vitro* model, confirming that mephedrone readily enters the brain (Hadlock *et al.*, 2011). Overall, the pharmacological effects of mephedrone and methylone appear to be relatively similar to those of MDMA but share more of the DA system-stimulating properties of amphetamine and methamphetamine and the DAT versus SERT inhibition profile of cocaine. The subjective effects of mephedrone have been reported to be similar to those of cocaine (Winstock *et al.*, 2011) but also MDMA (Carhart-Harris *et al.*, 2011). Importantly, MDMA is mostly used orally, whereas intranasal administration is the most common route of use for mephedrone (Winstock *et al.*, 2011) and cocaine. Users noted that the high obtained with the intranasal use of mephedrone was similar to or better than the high produced by cocaine (Winstock *et al.*, 2011). These observations indicate that the oral use of mephedrone produces overall similar effects to MDMA, whereas intranasal use results in more cocaine-like psychotropic effects. Similar to mephedrone and methylone, ethylone and butylone may be associated with an increased risk of addiction compared to their non-β-keto analogues because of the stronger relative activation of the DA system.

The pyrovalerone derivative naphyrone exhibited a monoamine uptake transporter inhibition profile that was very close to that of cocaine, with equal relative potency at all three transporters. Similar to cocaine, naphyrone was not a monoamine releaser. Naphyrone is distinct from pyrovalerone and its derivative MDPV because of its higher absolute and relative SERT-inhibiting potency. Although the structure would suggest similar pharmacological effects to the other pyrovalerone derivatives, the additional SERT inhibition may indicate more similar effects to cocaine in humans (Derungs *et al.*, 2011).

Methamphetamine-like cathinones

Cathinone and methcathinone exhibited a relative monoamine transporter inhibition profile that was very similar to that of the non-β-keto analogues amphetamine and methamphetamine, with high inhibitory potencies at the DAT and low potencies at the SERT, consistent with previous findings (Cozzi *et al.*, 1999; Fleckenstein *et al.*, 1999). Cathinone and methcathinone were also potent releasers of DA but not 5-HT, similar to amphetamine and methamphetamine. Cathinone and methcathinone have previously been shown to release radiolabelled DA and 5-HT from rat brain preparations with similar DA versus 5-HT selectivity to amphetamine (Kalix, 1990) and methamphetamine (Glennon *et al.*, 1987), but with two- to three-fold lower potency. Methcathinone has been shown to be a substrate for the transporter (Cozzi and Foley, 2003), similar to the classic amphetamines and MDMA (Rothman *et al.*, 2001; Verrico *et al.*, 2007). Cathinone and methcathinone produce amphetamine-like locomotor stimulation in animals (Glennon *et al.*, 1987; Kelly, 2011), and cathinone is self-administered by rats (Gosnell *et al.*, 1996) or rhesus monkeys (Johanson and Schuster, 1981; Woolverton and Johanson, 1984) with reinforcing efficacies comparable to amphetamine and cocaine. Clinically, cathinone and methcathinone have been reported to produce similar toxicity to amphetamine, including hypertension, hyperthermia, euphoria, locomotor activation and hallucinations following higher or repeated doses (Kalix, 1990; Widler *et al.*, 1994). Thus, the pharmacology of cathinone and methcathinone is qualitatively very close to that of amphetamine and methamphetamine.

Flephedrone was a DAT but not a SERT inhibitor, similar to its analogue 4-fluoroamphetamine (Nagai *et al.*, 2007). Flephedrone released DA but not 5-HT (IC_{50} >33 μM). The

DAT/SERT selectivity profile of flephedrone is therefore equal to the methamphetamine-like cathinones. In contrast, flephedrone had higher 5-HT_{2A} receptor binding that was similar to mephedrone and MDMA. We are not aware of any other *in vitro* or *in vivo* data on flephedrone. Agitation and psychosis were reported in a patient who insufflated flephedrone and MDPV powder (Thornton *et al.*, 2012).

Pyrovalerone cathinones

Pyrovalerone and its derivative MDPV were very potent DAT inhibitors as previously shown (Meltzer *et al.*, 2006) and at least 10-fold more potent than cocaine and methamphetamine. In contrast to the pyrovalerone derivative naphyrone, MDPV and pyrovalerone are weak inhibitors of the SERT, resulting in high DAT selectivity, with DAT/SERT inhibition ratios >100. MDPV and pyrovalerone were also the most potent NET inhibitors. Despite the high potency to block the DAT, pyrovalerone and MDPV did not produce DA efflux. Thus, pyrovalerone derivative cathinones are pure transporter uptake inhibitors. Consistent with the potent effect on catecholamine carriers, MDPV, compared with mephedrone, produced behavioural effects in animals at lower doses (Aarde *et al.*, 2011) and has been reported to produce mostly sympathomimetic toxicity and psychotic reactions in humans (Spiller *et al.*, 2011). Similar to methamphetamine, MDPV and pyrovalerone did not exhibit affinity for the 5-HT_{2A} receptor and exhibited low affinity for TA_1 receptors, consistent with the other cathinones.

Pyrovalerone derivatives have been suggested to easily cross the BBB because of their high lipophilicity (Meltzer *et al.*, 2006; Coppola and Mondola, 2012). All of the cathinones and non-β-keto amphetamine analogues showed good membrane permeation in our *in vitro* BBB model, but we indeed documented very high transmembrane permeability of MDPV and a potential active transport. Although the high brain uptake may contribute to the higher potency of MDPV compared with the non-pyrovalerone cathinones, the high potency at the NET and DAT is more likely to be responsible for the psychotropic effects at low doses in humans. In fact, we found that the potencies to inhibit the NET and DAT were significantly correlated with the doses reported to produce psychotropic effects in recreational users. Consistently, pyrovalerone and MDPV are at least 10-fold more potent inhibitors of the NET or DAT compared with mephedrone; this was demonstrated in the present study and previously (Meltzer *et al.*, 2006), and are used recreationally at approximately 10-fold lower doses than mephedrone (Derungs *et al.*, 2011), whereas both MDPV and mephedrone exhibited very high BBB penetrance in our study. The potency of the pyrovalerone derivatives at the DAT and NET and high brain penetrance could result in high sympathomimetic toxicity and risk of addiction in humans. MDPV has also been shown to be a potent reinforcer in rats, similar to methamphetamine (Watteron *et al.*, 2011).

Structure–activity relationship and binding to monoamine receptors

β-Keto-amphetamines appear to have similar effects on plasma membrane monoamine transporters compared with their non-β-keto analogues, with slightly higher selectivity for the DAT over the SERT. β-Keto-analogue cathinones also exhibited approximately 10-fold lower affinity for the TA_1 receptor compared with their respective non-β-keto amphetamines. TA_1 receptors play an important role in the modulation of dopaminergic and 5-hydroxytryptaminergic activity (Lindemann *et al.*, 2008; Revel *et al.*, 2011). Activation of TA_1 receptors negatively modulates dopaminergic neurotransmission. Importantly, methamphetamine decreased DAT surface expression via a TA_1 receptor-mediated mechanism and thereby reduced the presence of its own pharmacological target (Xie and Miller, 2009). MDMA and amphetamine have been shown to produce enhanced DA and 5-HT release and locomotor activity in TA_1 receptor knockout mice compared with wild-type mice (Lindemann *et al.*, 2008; Di Cara *et al.*, 2011). Because methamphetamine and MDMA auto-inhibit their neurochemical and functional effects via TA_1 receptors, low affinity for these receptors may result in stronger effects on monoamine systems by cathinones compared with the classic amphetamines. The higher selectivity of the cathinones for the DAT and lack of TA_1 receptor binding may result in an increased risk of dependence compared with classic non-β-keto analogue stimulants (Rothman and Baumann, 2006). Because 5-HT release dampens the stimulant effects of amphetamine-type drugs, the lower activity of the cathinones at the SERT would be expected to result in more stimulant-like effects (Baumann *et al.*, 2011) compared with the non-β-keto analogues. The α-ethyl-substituted compounds MBDB and butylone exhibited fivefold lower absolute and relative NET inhibition potencies than their α-methyl-analogues MDMA and methylone, in line with previous studies (Montgomery *et al.*, 2007). The lower affinity for NET has been associated with the low stimulant and euphorigenic properties of MBDB (Montgomery *et al.*, 2007).

Several of the drugs evaluated in the present study exhibited moderate direct affinity for 5-HT receptors and adrenoceptors, with K_i values in the 1–10 μM range. Direct interactions between MDMA and 5-HT_2 receptors rather than indirect agonist effects via 5-HT release have been suggested to contribute to MDMA-induced excitation and hallucinogen-like perceptual alterations at higher doses (Liechti *et al.*, 2000b; 2001). MDMA, MBDB, mephedrone, flephedrone and methcathinone bound to 5-HT_{2A} receptors, consistent with previous data on mephedrone, methylone and MDMA (Lopez-Arnau *et al.*, 2012; Martinez-Clemente *et al.*, 2012). Stimulation of 5-HT_{2A} receptors has also been shown to enhance DA release (Gudelsky *et al.*, 1994), potentially increasing abuse liability. Although flephedrone and methcathinone show low potency at the SERT, these drugs may have direct effects on the 5-HT system via 5-HT_{2A} receptor activation at higher doses. Amphetamine and methamphetamine bound to 5-HT_{1A} receptors, potentially resulting in behavioural effects that are opposite to those induced by 5-HT_{2A} receptor stimulation (Nichols, 2004; Gatch *et al.*, 2011). Mephedrone, flephedrone, cathinone and methcathinone exhibited affinity for α_{1A} adrenoceptors, which have been implicated in stimulant-induced vasoconstriction, hyperthermia (Hysek *et al.*, 2012b) and euphoria (Newton *et al.*, 2012). Finally, amphetamine and methamphetamine bound to α_{2A} receptors, which modulate NA release and sympathomimetic toxicity (Hysek *et al.*, 2012a).

It is important to note that we assessed the effects of racemic cathinones, whereas stereoselective and drug-specific interactions have been demonstrated for amphetamines (Lyon *et al.*, 1986; Steele *et al.*, 1987; Acquas *et al.*, 2007). Furthermore, we did not investigate drug interactions with intracellular targets such as monoamine oxidase or the vesicular monoamine transporter.

In summary, considerable differences were found in the pharmacology of the different cathinones. Mephedrone, methylone, ethylone, butylone and naphyrone acted as non-selective monoamine uptake inhibitors, similar to cocaine and, with the exception of naphyrone, also induced the release of 5-HT, similar to MDMA. Cathinone and methcathinone were found to be selective catecholamine uptake inhibitors and releasers, similar to their non-β-keto analogues amphetamine and methamphetamine. Pyrovalerone and MDPV were shown to be highly potent and selective catecholamine transporter inhibitors but not substrate releasers.

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Conflict of interest

None.

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Paper 1b

Monoamine Transporter and Receptor Interaction Profiles of a New Series of Designer Cathinones

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RESEARCH PAPER

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

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Summary

Background and purpose: 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.

Experimental approach: We determined noradrenaline (NA), dopamine (DA), and serotonin (5-HT) uptake inhibition using HEK 293 cells that express the respective human monoamine transporter. We investigated drugs-induce efflux of NE, DA, or 5-HT from monoamine-preloaded cells and determined the binding affinities to monoamine transporters and receptors.

Key results: All cathinones were potent NA uptake inhibitors, but differed in their DA versus 5-HT transporter inhibition profiles and in their effects to release monoamines. Methedrone was a more potent 5-HT than DA transporter inhibitor and released NA and 5-HT similar to para-methoxymethamphetamine (PMMA), para-methoxyamphetamine (PMA), 4-methylthioamphetamine (4-MTA), or 3,4-methylenedioxymethamphetamine (MDMA). 4-MEC and pentylone equipotently inhibited all monoamine transporters and released 5-HT. Ethcathinone and 3-FMC inhibited NA and DA uptake and released NA and 3-FMC also DA similar to N-ethylamphetamine and methamphetamine. Pentedrone and N,N-dimethylcathinone were non-releasing NA and DA uptake inhibitors as previously shown for pyrovalerone cathinones. Buphedrone preferentially inhibited NA and DA uptake and also released NA. 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.

Conclusions and implications: We described considerable differences in the monoamine transporter interaction profiles among different cathinones and compared with related amphetamines.

Keywords: cathinone, amphetamine, legal high, research chemical, serotonin, dopamine, noradrenaline transporter

Abbreviations

DA, dopamine; DAT, dopamine transporter; HEK, human embryonic kidney; 5-HT, serotonin; MDMA, 3,4-methylenedioxymethamphetamine; MDPV, 3,4-methylenedioxypyrovalerone; 4-MTA, 4-methylthioamphetamine; NA, noradrenaline; NET, noradrenaline transporter; PMA, para-methoxyamphetamine (PMA), PMMA, para-methoxymethamphetamine; SERT, serotonin transporter; TA, trace amine-associated receptor

Introduction

The market of illicit stimulants has become complex. From 2005 to 2011, 34 novel cathinone-type designer drugs have been detected in the European Union (EMCDDA, 2012). These drugs are typically available online as "legal highs", "bath salts", or "research chemicals" (EMCDDA, 2012) and added to the users' club drug repertoires (Moore *et al.*, 2013) resulting in unknown health risks. Intoxications with different cathinone derivatives have been reported worldwide (Borek & Holstege, 2012; James *et al.*, 2011; Prosser & 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 to compare the pharmacological mechanism of action of the novel designer drugs with well-known amphetamines including methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA).

In the present study, we characterized the *in vitro* pharmacology of a second series of cathinones including methedrone, 4-methylethcathinone (4-MEC), 3-fluoromethcathinone (3-FMC), pentylone, ethcathinone, buphedrone, pentedrone, and N,N-dimethylcathinone, as well as profiles of the non-β-keto amphetamine analog comparator drugs 4-methylthioamphetamine (4-MTA), para-methoxyamphetamine

(PMA), para-methoxymethamphetamine (PMMA), MDMA, N-ethylamphetamine, and methamphetamine, complementing our previous characterization of this class of designer drugs (Simmler *et al.*, 2013). Being 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 noradrenaline (NA), dopamine (DA), and serotonin (5-HT) uptake transporters (NET, DAT, and SERT, respectively). We tested whether the drugs induce transporter-mediated release of NA, DA or 5-HT and characterized the biniding affinities of the drugs for monoamine transporters, α_1 and α_2 adrenergic receptors, dopamine $D_{1\text{-}3}$ receptors, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} receptors, the histamine H_1 receptor, and the trace amine-associated receptor 1 (TA_1).

Methedrone is the β-keto-substituted analog of PMMA. PMMA and 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 for many years worldwide (Johansen *et al.*, 2003; Lurie *et al.*, 2012; Vevelstad *et al.*, 2012). PMA and PMMA use has been associated with particularly high morbidity and mortality in particular due to hyperthermia (Brunt *et al.*, 2012; Lurie *et al.*, 2012; Refstad, 2003). Methedrone is found in bath salts products (Marinetti & Antonides, 2013) and it may exhibit a high risk for mortality (Wikstrom *et al.*, 2010) similar to PMA. PMA inhibits the SERT and induces 5-HT release (Callaghan *et al.*, 2005) like MDMA. In animals, PMMA and PMA produce effects similar to MDMA, but 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 not available. 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 & Byrska, 2012). 4-MEC inhibits the DAT, NET, and SERT (Iversen *et al.*, 2013). Whether 4-MEC is also a monoamine releaser is not known.

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 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 presenting 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 NA and 5-HT, but not DA from rat synaptosomes (Yu *et al.*, 2000). N-Ethylamphetamine releases NA and with lower potency also 5-HT and DA from rat synaptosomes (Tessel & Rutledge, 1976). We found no data on effects of ethcathinone on the human monoamine transporters.

Pentedrone, buphedrone, and pentylone are recently identified novel designer cathinones (Maheux & Copeland, 2012; Westphal *et al.*, 2012; Zuba & Byrska, 2012). Buphedrone was detected in "Vanilla Sky" and other "legal high" pills as a frequent substitute for mephedrone in Eastern Europe (Zuba *et al.*, 2013). Intoxications 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 headshops (Westphal *et al.*, 2012) or fatalities associated with "bath salts" in the US (Marinetti & 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 (Figure 1). Pentylone is a β-keto-analog of MDMA, similar to methylone and butylone (Simmler *et al.*, 2013), containing 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.

Methods

Drugs

Drugs were supplied by Lipomed (Arlesheim, Switzerland) as hydrochloride salts (purity >98.5%). Racemic drugs were used except for d-methamphetamine. All 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).

Monoamine uptake transporter inhibition

Inhibition of NET, SERT, and DAT was assessed in HEK 293 cells that stably expressed human NET, SERT or DAT (Tatsumi *et al.*, 1997) as previously described (Hysek *et al.*, 2012). DAT/SERT ratio was calculated as 1/DAT IC_{50} : 1/SERT IC_{50}.

Transporter-mediated monoamine release

We studied transporter-mediated NA, 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). In brief, 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]NA and 10 µM unlabeled NA for 20 min. Then, we washed twice, and induced release by adding 1000 µM of release buffer containing the test drugs at concentrations of 10 µM and 100 µM. 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 r.p.m on a rotary shaker. We then stopped the release by removing the buffer and gently washing the cells twice with cold buffer. We quantified the radioactivity remaining in the cells (HEK-SERT and HEK-DAT) or in the removed buffer (HEK-NET). Nonspecific "pseudoefflux", which arises from substrate diffusing out of the cells and inhibition of reuptake (Rosenauer *et al.*, 2013; Scholze *et al.*, 2000), was assessed using the transporter inhibitors nisoxetine (HEK-NET cells), citalopram (HEK-SERT cells), or mazindol (HEK-DAT cells) at 10 µM. Nonspecific release was subtracted from E_{max} of total release to yield E_{max} values of specific transporter-mediated release. Methamphetamine and MDMA were used as comparator compounds known to induce monoamine release in this assay (Simmler *et al.*, 2013). We used analysis of variance followed by Dunnett's tests to compare drug

effects with the control condition. Drugs that induced significantly higher maximal monoamine efflux compared to the respective transporter inhibitors (nonspecific release) were considered monoamine releasers.

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 TA_1 receptors that were rat/mouse) (Revel *et al.*, 2011) were incubated with the radiolabelled selective ligands at concentrations equal to K_d and ligands displacement by the compounds measured. The following radioligands and competitors were used: *N*-methyl-[^{3}H]-nisoxetine and indatraline (NET), [^{3}H]citalopram and indatraline (SERT), and [^{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 (TA1). To evaluation the relationship between binding affinity to the transporter and functional inhibition potencies, we calculated Pearson correlations for the pure transporter inhibitor and substrate releaser subgroups.

Results

Monoamine uptake transporter inhibition

The effects of the cathinones and comparator drugs on monoamine transporter function are shown in Figure 1. The IC_{50} values for monoamine transport inhibition and the 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 compounds shared potent effects as NET inhibitors while their DAT and SERT inhibition

potencies varied considerably as evidenced by the wide range of their DAT/SERT inhibition ratios. 4-MTA, PMA, PMMA, and methedrone were potent SERT and NET inhibitors but weak inhibitors of DAT similar to MDMA. The DAT/SERT ratio was below 0.5 for all these MDMA-like drugs. Methedrone was the only cathinone in the present series of drugs with an MDMA-like profile and DAT/SERT ratio below 1. With the exception of methedrone, all cathinones examined in this series preferentially inhibited NET and DAT over SERT. In particular 3-FMC, pentedrone, buphedrone, and N,N,-dimethylcathinone exhibited very low potency at SERT and DAT/SERT inhibition ratios >10. Ethylamphetamine was equipotent at DAT and SERT, while its β-keto analog ethcathinone was 10-fold less potent at SERT versus DAT. In contrast, 4-MEC, the 4-ring-methylated analog of ethcathinone, was 6-fold more potent at SERT than ethcathinone. Similarly, the 3,4-ring methoxylated analog of pentedrone, pentylone was at least 10-fold more potent at SERT than pentedrone. NET, but not DAT or SERT inhibition potency values (IC_{50}) were correlated with estimated reported mean doses taken by recreational users (http://www.erowid.org and other user home pages) (R=0.60; P<0.05, N=13; Table 1). N,N-dimethylcathinone was not included due to a lack of data on effective doses used in humans.

Transporter-mediated monoamine release

Maximal monoamine releasing effects are shown in Figure 2. All compounds induced release of at least one monoamine with the exception of pentedrone and N,N-dimethylcathinone, which were non-releasers. Methedrone and 3-FMC released all three monoamines similar to all the non-β-keto amphetamines methamphetamine, MDMA, PMMA, PMA, and N-ethylamphetamine. Ethcathinone and 4-MTA released NA and 5-HT. 4-MEC and pentylone weakly released 5-HT. Buphedrone released NA.

Binding affinities

Monoamine transporter and receptor binding affinities are shown in Table 2. None of the drugs exhibited very high binding (<100 nM) to any of the monoamine transporters or human receptors. There were submicromolar (<1 μM) affinity DAT interactions with the cathinones 4-MEC, pentylone, ethcathinone, and pentedrone but

not with the non-β-keto amphetamines. Conversely, the non-β-keto amphetamines but not the cathinones showed affinity to rat and mouse TA.

Ethcathinone and the non-β-keto amphetamines N-ethylamphetamine and methamphetamine showed relevant (<10 μM) 5-HT_{1A} receptor binding. The cathinones 4-MEC and N,N-dimethylcathinone and the non-β-keto amphetamines 4-MTA and MDMA showed relevant (<10 μM) 5-HT_{2A} receptor binding. The cathinones 4-MEC, 3-FMC, and N,N-dimethylcathinone and the non-β-keto amphetamines 4-MTA and N-ethylamphetamine showed relevant (<10 μM) 5-HT_{2C} receptor binding. 4-MTA, N-ethylamphetamine, and methamphetamine showed relevant (<10 μM) adrenergic α_2 receptor binding. None of the drugs tested bound to DA D_{1-3} or histamine H_1 receptors.

There were differences in the associations between transporter affinities and functional transporter inhibition potencies between the pure uptake inhibitors and the monoamine releasers depending on transporter type. For the NET, K_i and IC_{50} values were significantly correlated for the pure transporter inhibitors ($R=0.97$, $P<0.05$, $N=4$) but not for the releasers ($R=0.08$, NS, $N=8$). For the DAT, K_i and IC_{50} values were significantly correlated for both the pure transporter inhibitors ($R=0.93$, $P<0.001$, $N=8$) and releasers ($R=0.87$, $P<0.05$, $N=7$). For the SERT, K_i and IC_{50} values were not correlated for the pure transporter inhibitors nor the releasers ($R=0.53$, NS, $N=3$ and $R=0.6$, NS, $N=10$; respectively). Additionally, the pure uptake inhibitors and releasers typically differed by their ratio of K_i value to IC_{50} value. Most releasers showed higher functional potency values compared to binding potency values resulting in ratios > 6 for NET and >1 for DAT and SERT with some exceptions for drugs that released monoamines only at high drug concentrations (>10 μM).

Discussion

We characterized the monoamine receptor binding profiles and interactions with the monoamine transporters of a series of seven novel designer cathinones, 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 cathinones inhibited monoamine transport as expected

based on their amphetamine structure. However, there are considerable differences among these cathinones as well as in comparison with the non-β-keto amphetamines. We have previously characterized a first series of ten cathinone derivatives together with five non-β-keto amphetamine analogs and cocaine using identical methods as in the present study (Simmler *et al.*, 2013). The profiles of the second series of compounds described here can therefore directly be compared with those of our previous study (Simmler *et al.*, 2013), which have been confirmed and extended by others (Baumann *et al.*, 2013; Eshleman *et al.*, 2013; Iversen *et al.*, 2013; Rosenauer *et al.*, 2013). First, the compounds markedly differed with regard to their serotoninergic versus dopaminergic properties as expressed by the DAT/SERT ratio, possibly an *in vitro* marker of the abuse potential of a drug (Rothman & Baumann, 2006; Simmler *et al.*, 2013). Second, some cathinones were pure uptake inhibitors while others were substrate releasers. Third, cathinones typically did not bind to rodent TA1 in contrast to the non-β-keto-amphetamines.

Methedrone is a serotonergic cathinone similar to other para-(4)-substituted amphetamines and MDMA

Methedrone was 7-fold more potent as a SERT versus DAT inhibitor and was the cathinone with the highest selectivity for SERT in both the present and previously studied series of compounds (Simmler *et al.*, 2013), exhibiting a profile identical to MDMA. Methedrone also produced monoamine efflux similar to MDMA. Methedrone is therefore the most MDMA-like cathinone presently described in terms of monoamine transporter interaction profile. Interestingly, methylone, which is the exact β-keto analog of MDMA, was less selective for the SERT versus DAT compared to methedrone (Simmler *et al.*, 2013). Methedrone is the β-keto analog of PMMA. Both methedrone and PMMA are para-methoxy- or methylthio-amphetamines similar to PMA and 4-MTA, which have been included as comparator compounds into the present series. PMA is the demethylated analog of PMMA and also a metabolite of PMMA in vivo (Staack & Maurer, 2005). Compared to other amphetamines, PMA, PMMA, and 4-MTA have long been associated with particularly high clinical toxicity and many fatalities mostly due 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). Similar fatal intoxications have

recently been described with methedrone (Wikstrom *et al.*, 2010). Our study provides first molecular pharmacological data on methedrone and PMMA and confirms the previously characterized profiles of PMA and 4-MTA (Gobbi *et al.*, 2008; Gough *et al.*, 2002; Huang *et al.*, 1992; Quinn *et al.*, 2006). The present study showed that all these para-substituted amphetamines are potent NET and SERT inhibitors with low potency at DAT confirming data on 4-MTA (Huang *et al.*, 1992). The compounds have similar or even higher preference for SERT over DAT compared with MDMA. They all released NA and 5-HT and at high concentrations (>10 μM) also DA as previously shown for 4-MTA and PMA in vitro and in vivo (Gobbi *et al.*, 2008; Gough *et al.*, 2002; Huang *et al.*, 1992; Quinn *et al.*, 2006; Sotomayor-Zarate *et al.*, 2012). 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), as well as monoamine oxidase A (MAO) inhibition (Carmo *et al.*, 2003). In fact, PMA is a much stronger MAO inhibitor than other amphetamines (Green & El Hait, 1980).

4-MEC and pentylone are cocaine-MDMA-mixed cathinones

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

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 NET and DAT than SERT compared with their non-β-keto amphetamine analogs (Iversen *et al.*, 2013; Simmler *et al.*, 2013). Ethcathinone and 3-FMC exhibited similar monoamine uptake transporter inhibition profiles to methamphetamine and flephedrone (Simmler *et al.*, 2013). In addition, ethcathinone released NA and 5-HT but not DA as shown previously in studies using rat synaptosomes (Yu *et al.*, 2000) and 3-FMC released all monoamines. Similar release of DA and 5-HT has also been documented for flephedrone, the positional isomer of 3-FMC (Simmler *et al.*, 2013). Ethcatinone and 3-FMC can be classified as a methamphetamine-like cathinones (Simmler *et al.*, 2013) although ethcathinone did not release DA.

Pentedrone and N,N-dimethylcathinone are pure monoamine uptake inhibitors similar to the pyrovalerone cathinones

Pentedrone and N,N-dimethylcathinone are uptake inhibitors only similar to cocaine and as previously shown for 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 the substrate-type releasers (Eshleman *et al.*, 2013). All non-releaser compounds including pentedrone and N,N-dimethylcathinon and the pyrovalerones pyrovalerone, MDPV, and naphyrone (Simmler *et al.*, 2013) are tertiary amines or 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 transporter NET and DAT versus SERT, although with lower potency and catecholamine transporter selectivity compared with pyrovalerone and MDPV, which are very potent and selective catecholamine inhibitors (Simmler *et al.*, 2013). 4- or 3,4-Substitutions at the phenyl ring result in serotonergic selectivity as documented by the low DAT/SERT inhibition ratio of MDMA, 4-MTA, PMA, PMMA, and methedrone. Similarly, the 4- or 3,4-phenylringsubstituted compounds methylethcathinone, pentylone, and naphyrone (Simmler *et al.*, 2013) have some

activity at the SERT. None of the cathinone derivatives characterized in the present study exhibited the very high potency at the DAT and the high DAT/SERT inhibition ratio > 100 previously shown for MDPV (Baumann *et al.*, 2013; Simmler *et al.*, 2013) and associated with compulsive use (Watterson *et al.*, 2012). Nevertheless, the DAT/SERT inhibition ratios of buphedrone, pentedrone, and N,N-dimethylcathinone were all > 10, similar to methamphetamine (Simmler *et al.*, 2013), and possibly indicating high abuse potential (Bauer *et al.*, 2013). NET inhibition potency was inversely associated with the reported doses of the drugs abused by humans, consistent with our previous findings (Simmler *et al.*, 2013). This correlation was not found for DAT or SERT inhibition potencies. N,N-dimethylcathinone was not added to the correlation because of its low transporter inhibition potency values and the lack of data on dosing in humans. The monoamine transporter inhibition potencies of N,N-dimethylcathinone were at east 10-fold lower than the ones of d-amphetamine or d-methamphetamine in the present study. We are not aware of other *in vitro* data. In animal tests of stimulus generalization, racemic N,N-dimethylcathinone was 1.5 fold less potent than d-amphetamine or d-methamphetamine (Dal Cason *et al.*, 1997). The reason for this discrepancy is not clear.

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

Binding profiles to monoamine receptors and transporters

In the present study, we also evaluated binding to the monoamine transporters and to a series of other monoaminergic receptors. 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 clinical effects of these drugs. We have previously shown that cathinones exhibit approximately 10-fold lower affinity for TA_1 receptors compared to their non-β-keto analogs (Simmler *et al.*, 2013). We have now extended this observation by showing that a series of additional cathinones consistently did not show relevant TA_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 or methamphetamine inhibit their own neurochemical and locomotor stimulant effects via TA_1 activation (Di Cara *et al.*, 2011). The lack of this TA_1-mediated "auto-inhibition" in the cathinones may contribute to more stimulant-like and addictive properties in this new class of designer drugs compared with the 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 here showed low-affinity binding to these receptors. N-Ethylamphetamine, ethcathinone, and methamphetamine bound to 5-HT_{1A} receptors as observed for some other compounds from our previous series (Simmler *et al.*, 2013). The cathinones 4-MEC and N,N-dimethylcathinone exhibited affinity to 5-HT_{2A} receptors similar to the non-β-keto amphetamines MDMA and 4-MTA, and as previously shown for the cathinones mephedrone, flephedrone, naphyrone, and methcathinone (Eshleman *et al.*, 2013; Simmler *et al.*, 2013). The cathinones 4-MEC, 3-FMC, and N,N-dimethylcathinone as well as the non-β-keto amphetamines 4-MTA and N-ethylamphetamine bound to 5-HT_{2C} receptors. In line with our previous study (Simmler *et al.*, 2013) and the work by others (Eshleman *et al.*, 2013), no submicromolar binding was observed at the 5-HT_{1A}, 5-HT_{2A}, or 5-HT_{2C} receptors for any of the drugs. Further, 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 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 to the 5-HT_{2B} receptor 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 fibroses (Roth, 2007) as well as in the behavioral effects of MDMA (Doly *et al.*, 2008). Some previously described cathinones but none of the present ones exhibited low micromolar affinity for adrenergic α_1 receptors. Low-affinity α_2 receptors binding was observed for most non-β-keto amphetamines and some of the cathinones in the present and in our previous study (Simmler *et al.*, 2013). There were no relevant interactions with DA $D_{1\text{-}3}$ or histamine H_1 receptors.

Substrate releasers show higher transporter inhibition potency values compared to binding affinity values, resulting in ratios of binding K_i to uptake inhibition IC_{50} >1 (Eshleman *et al.*, 2013; Rudnick & Wall, 1992; Simmler *et al.*, 2013). This phenomenon was also observed for most but not all monoamine releasers in the present study. Additionally, it has previously been noted that binding potencies correlate poorly with the inhibition potencies of substrate uptake (Iversen *et al.*, 2013). We found significant correlations between DAT binding and DAT inhibition potencies for both the pure transporter inhibitors and the substrate releasers across the 14 drugs studied here or across 27 drugs including also the previously characterized drugs (Simmler *et al.*, 2013). In contrast, we found no associations between SERT binding and SERT inhibition potencies. For the NET, binding and inhibition potencies were correlated only for the pure uptake inhibitors, but not for the releasers. These findings may indicate that radioligands used in the SERT binding assay and SERT inhibitors likely bind to different sites on the SERT (Iversen *et al.*, 2013). In contrast, radioligands and pure transporter inhibitors, but not amphetamine-type catecholamine releasers, may bind to the same sites at the DAT and the NET.

The present study has limitations. First, 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. Second, we did not investigate drug effects on intracellular targets such as the vesicular monoamine transporter or monoamine oxidase, which are typically affected by amphetamines (Eshleman *et al.*, 2013; Green & El Hait, 1980). Of note, no relevant interactions with the vesicular monoamine transporter were observed for a series of first-generation cathinones (Eshleman *et al.*, 2013).

In summary, we described the pharmacology of a series of novel designer cathinones. These drugs were all potent inhibitors of NET, but there were marked differences in their DAT and SERT inhibition profiles and in their properties to also release monoamines.

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Conflict of interest

None.

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Table 1 Monoamine transporter inhibition

	NET	DAT	SERT	DAT/SERT ratio	Recreational dose**
	IC50 [µM] (95% CI)	IC50 [µM] (95% CI)	IC50 [µM] (95% CI)	Ratio (95% CI)	mg
4-MTA*	1.52 (1.3-1.9)	22 (15-32)	0.54 (0.37-0.80)	0.02 (0.01-0.05)	120
PMA*	0.80 (0.50-1.0)	71 (60-83)	2.37 (2.0-2.9)	0.03 (0.02-0.05)	70
PMMA*	1.20 (0.75-1.8)	49 (18-135)	1.77 (1.1-2.9)	0.04 (0.01-0.16)	50
MDMA*,#	0.45 (0.33-0.60)	17 (12-24)	1.36 (1.0-2.0)	0.08 (0.04-0.16)	100
Methedrone	2.24 (1.4-3.5)	35 (15-79)	4.73 (3.2-6.9)	0.14 (0.04-0.46)	200
N-Ethylamphetamine*	0.20 (0.15-0.27)	5.86 (4.8-7.1)	8.77 (6-13)	1.5 (0.9-2.7)	100
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)	100
Pentylone	0.99 (0.72-1.4)	1.34 (1.0-1.7)	8.37 (5.4-13)	6.2 (3.2-13)	125
Ethcathinone	0.44 (0.34-0.56)	5.00 (3.7-6.8)	48 (4-529)	9.6 (0.6-142)	60
Methamphetamine*,#	0.064 (0.04-0.09)	1.05 (0.74-1.5)	23 (14-40)	> 10	30
3-FMC	0.19 (0.13-0.29)	1.7 (1.0-3.0)	56 (7-472)	>10	100
Buphedrone	0.65 (0.51-0.81)	4.24 (3.3-5.5)	70 (2-2700)	> 10	80
Pentedrone	0.61 (0.52-0.72)	2.50 (2.0-3.2)	135 (5-3700)	> 10	100
N,N-Dimethylcathinone	7.71 (5-12)	27 (21-36)	> 500	> 10	NA

Values are means of three to four independent experiments and 95% confidence intervals (CI).
Drugs are ranked according to the DAT/SERT ratio = 1/DAT IC_{50} : 1/SERT IC_{50}.
*non-beta-keto-amphetamine comparator compounds.
**estimated average.
values from Simmler et al.
NA, not available

Table 2. Monoamine transporter and receptor binding affinities

	NET	DAT	SERT	5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	a_{1A}	a_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$
4-MTA*	1.2±0.6	6.8±1.3	28.0±15	>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*	15.6±2.1	18.5±3.9	7.1±0.8	>20	11.2±2.1	>13	>6	>25	>12.5	6.4±4.0	>16	>13	0.66±0.01	0.14±0.1
PMMA*	13.7±6.0	24.5±3.9	11.7±1.4	>20	13.6±3.0	>13	>6	>25	>12.5	10.1±1.0	14.0±3.4	>13	1.25±0.2	0.26±0.1
MDMA*,#	30.5±8.0	6.5±2.5	13.3±0.6	12.2±0.8	7.8±2.4	>13	>6	15.0±10	>12.5	25.2±12	>16	>13	0.37±0.12	2.4±1.1
Methedrone	15.6±4.6	20.7±3.4	25.7±14	>20	>13	>13	>6	>25	>12.5	>10	>16	>13	>10	>10
N-Ethylamphetamine*	>30	1.0±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±1.6	>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	>13	>6	15.5±1.9	>12.5	>10	>16	>13	>10	>10
Methamphetamine*,#	4.3±2.1	1.9±0.9	26.7±11	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	>30	1.9±0.2	36.1±1.4	>18	>13	6.1±2.2	>6	10±2.2	>12.5	>10	>16	>13	>10	5.8±4.0
Buphedrone	4.7±3.6	1.2±0.2	29.2±18.5	>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)
*non-beta-keto-amphetamine comparator compounds.
#values for MDMA are from Simmler et al. 2013

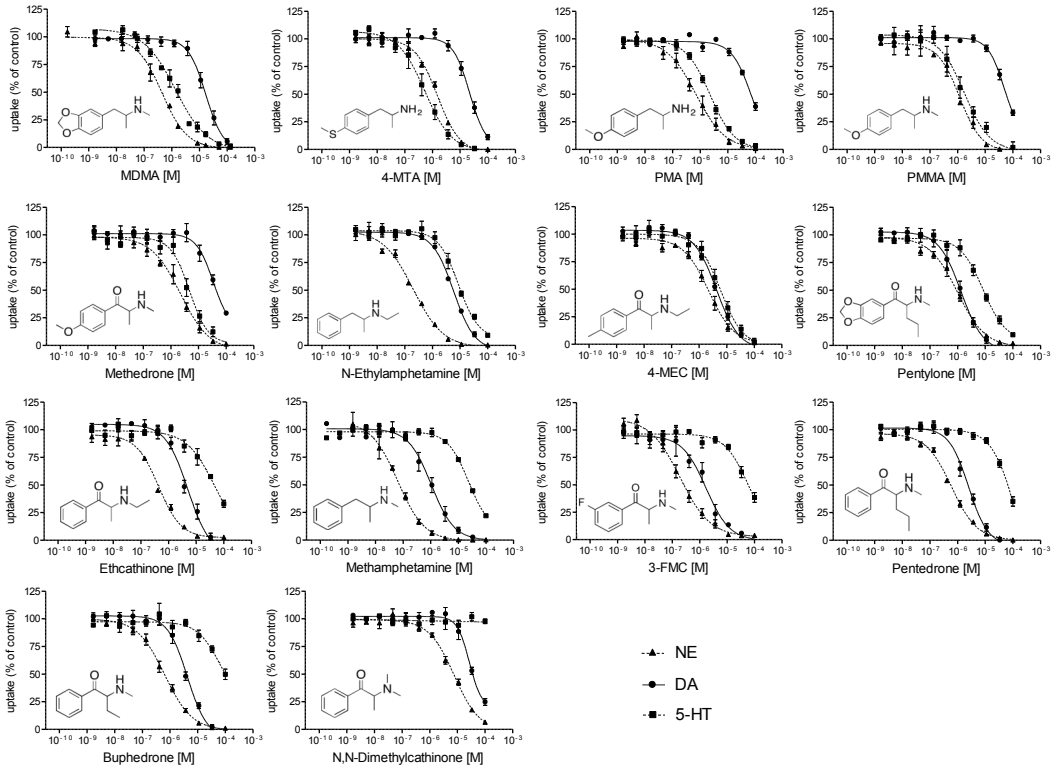

Figure 1 Monoamine uptake inhibition. Dose-response curves for the inhibition of [^{3}H]NA, [^{3}H]DA, and [^{3}H]5-HT into NET-, DAT-, and SERT-transfected HEK 293 cells, respectively. The data are expressed as means ± SEM of three independent experiments. The lines show the data fit by nonlinear regression. IC_{50} values are shown in Table 2.

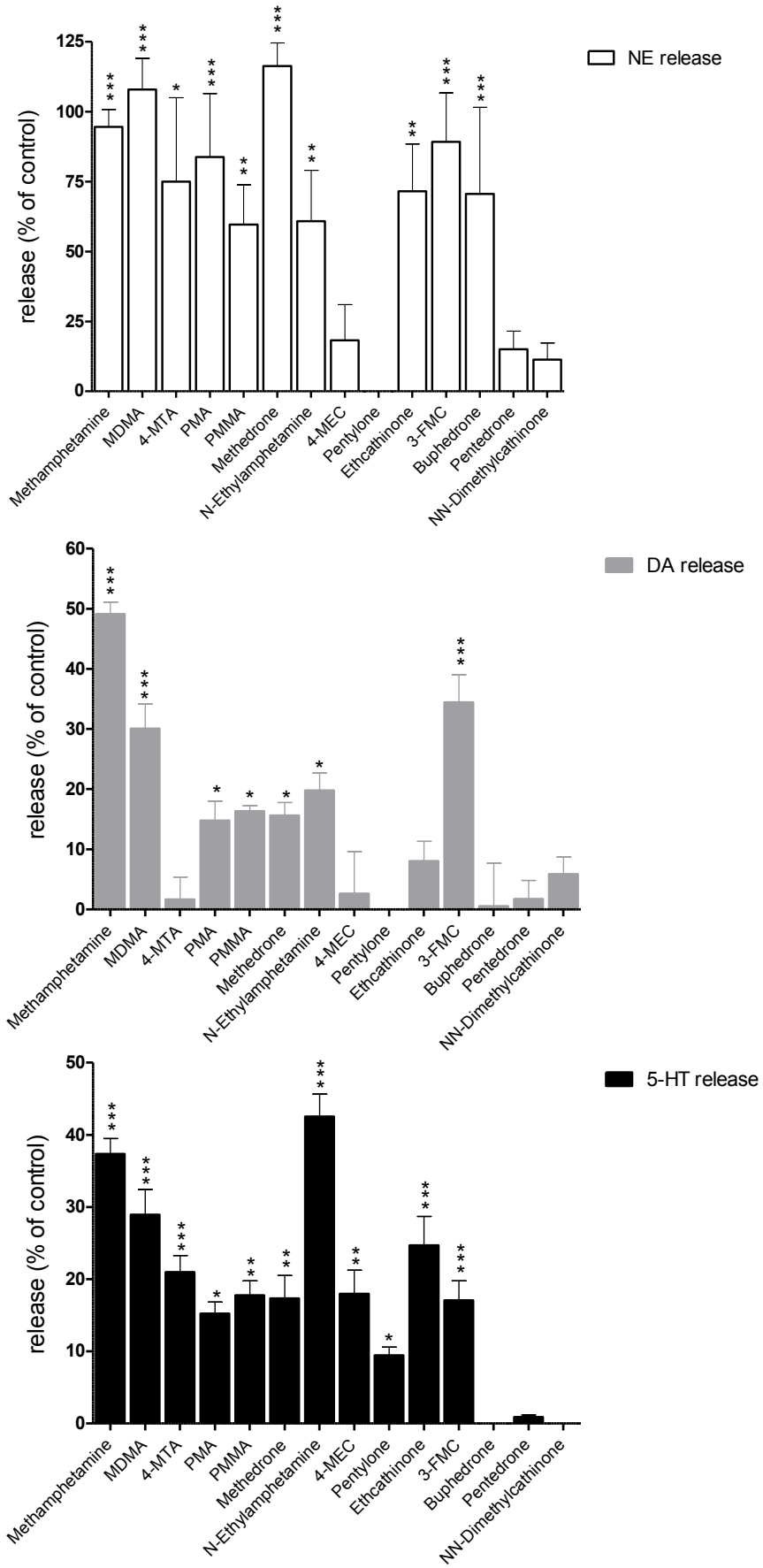
NE release
release (% of control)
125
100
75
50
25
0
Methamphetamine
MDMA
4-MTA
PMA
PMMA
Methedrone
N-Ethylamphetamine
4-MEC
Pentylone
Ethcathinone
3-FMC
Buphedrone
Pentedrone
NN-Dimethylcathinone
DA release
release (% of control)
60
50
40
30
20
10
0
5-HT release
release (% of control)
50
40
30
20
10
0

Figure 2 Monoamine release. HEK 293 cells expressing NET, DAT, or SERT were loaded with [^{3}H]NA, [^{3}H]DA, or [^{3}H]5-HT, respectively, washed and incubated with drugs. Monoamine release is expressed as the maximal release in % of controls at high drug concentrations (10 μM or 100 μM). The data are expressed as the mean ± SEM of at least three independent experiments. *P<0.05, **P<0.01, and ***P<0.001, significant effects compared with controls.

Chapter 2: Acute effects of MDMA in humans – mechanistic studies

Chapter 2 focuses on the acute psychostimulant effects in humans, taking the empathogen amphetamine-derivative MDMA as model substance. We investigated the role of transporter-mediated NE and 5-HT release in the cardio- and psychostimulant effects of MDMA in paper 2a and paper 2b. We addressed this objective in two randomized placebo-controlled studies with healthy volunteers by assessing the interaction between a pure transporter blocker and MDMA. Paper 2b also contains in vitro data that describe and confirm the interaction of MDMA with the transporter blocker at the isolated targets. Paper 2a, assessing the role of NE release alone, and paper 2b, describing the role of the NET/SERT double-blockade, are presented here as synergistic pieces of work.

Paper 2c has its focus on MDMA-induced increases in antidiuretic hormone that could result in hyponatremia and ensuing life-threatening complications. This study also investigates the role of NE-/5-HT release in MDMA-induced increased antidiuretic hormone plasma levels and points toward differences in men and women.

Content of chapter 2:

Paper 2a: The norepinephrine transporter inhibitor reboxetine reduces stimulant effects of MDMA (“Ecstasy”) in humans

Paper 2b: Duloxetine inhibits effects of MDMA (“Ecstasy”) in vitro and in humans in a randomized placebo-controlled laboratory study

Paper 2c: Sex differences in the effects of MDMA (“Ecstasy”) on plasma copeptin in healthy subjects

Paper 2a

The Norepinephrine Transporter Inhibitor Reboxetine Reduces Stimulant Effects of MDMA ("Ecstasy") in Humans

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This study assessed the pharmacodynamic and pharmacokinetic effects of the interaction between the selective norepinephrine (NE) transporter inhibitor reboxetine and 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") in 16 healthy subjects. The study used a double-blind, placebo-controlled crossover design. Reboxetine reduced the effects of MDMA including elevations in plasma levels of NE, increases in blood pressure and heart rate, subjective drug high, stimulation, and emotional excitation. These effects were evident despite an increase in the concentrations of MDMA and its active metabolite 3,4-methylenedioxyamphetamine (MDA) in plasma. The results demonstrate that transporter-mediated NE release has a critical role in the cardiovascular and stimulant-like effects of MDMA in humans.

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is widely used as a recreational drug, but it is also being investigated as an adjunct to psychotherapy in patients with post-traumatic stress disorder.[1] In humans, MDMA produces euphoria, happiness, and cardiovascular activation.[2–4] *In vitro*, MDMA induces carrier-mediated release of dopamine (DA), serotonin (5-HT), and norepinephrine (NE) through DA (DAT), 5-HT (SERT), and NE transporter (NET), respectively.[5–9] However, it is not clear how these monoamines contribute to the acute psychostimulant effects of MDMA in humans.[8,10]

The role of DA in the reinforcing effects of psychostimulants is well established in animal models. However, deletions of dopamine D_1, D_2, and D_3 receptor genes in mice had minimal effects on MDMA-induced acute changes in locomotor behavior,[11] and DAT inhibition did not affect acute responses to MDMA in rhesus monkeys.[12] In humans, DA D_2 receptor antagonists reduced amphetamine-induced and MDMA-induced euphoria only at doses that produced dysphoria.[13–15] Therefore, non-DA systems may be principally responsible for the acute effects of MDMA.

SERT inhibitors (SSRIs) decrease MDMA-induced 5-HT release *in vitro*[7] and in animals[16] and also attenuate behavioral effects of MDMA in animals.[17] Consistent with this preclinical evidence for a role of SERT, SSRIs reduced the subjective and cardiovascular response to MDMA in humans,[18–21] indicating that MDMA-induced, SERT-mediated 5-HT release critically contributes to the psychotropic and physical effects of MDMA in humans. However, the blood pressure response to MDMA is only partly attenuated by blockade of 5-HT release[18] and is largely unaffected by postsynaptic 5-HT_1 or 5-HT_2 receptor antagonist pretreatment.[22,23]

The role of the NET in the mechanism of action of MDMA in humans has not yet been explored. As compared to SERT and DAT, MDMA exhibits higher affinity for human NET.[5,6] MDMA releases NE more potently than 5-HT or DA from monoamine-preloaded human embryonic kidney (HEK) cells transfected with the corresponding human monoamine transporter.[6] The NET inhibitor desipramine and the SERT inhibitor citalopram, but not the DAT/NET inhibitor methylphenidate, reversed the acute cognitive effects of MDMA in rhesus monkeys.[12] NE also plays a role in mediating the peripheral effects of MDMA. MDMA increases the levels of circulating NE in rats.[24] The adrenergic α_1 receptor antagonist prazosin reversed MDMA-associated locomotor stimulation[25] and vascular effects[26] in rats. The NET inhibitor nisoxetine abolished contraction of the rat aorta produced by 4-methylthioampethamine,[27] a compound with a pharmacology similar to that of MDMA. Clinically, MDMA increases plasma NE levels[4] and stimulates

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Table 1 Mean ± SEM values and statistics of drug effects

		Placebo–placebo (mean ± SEM)	Reboxetine–placebo (mean ± SEM)	Placebo–MDMA (mean ± SEM)	Reboxetine–MDMA (mean ± SEM)	F(3,45) =	P <
Circulating catecholamines							
Epinephrine (nmol/l)	At 1 h	0.11 ± 0.02	0.08 ± 0.02	0.35 ± 0.08*	0.21 ± 0.03	6.70	0.001
Norepinephrine (nmol/l)	At 1 h	1.29 ± 0.16	1.18 ± 0.15	2.05 ± 0.20**	1.31 ± 0.13††	12.40	0.001
Physiologic effects							
SBP (mm Hg)	E_{max}	11.1 ± 2.1	8.5 ± 1.5	38.7 ± 2.6***	20.7 ± 2.6*,†††	33.50	0.001
DBP (mm Hg)	E_{max}	8.6 ± 1.5	5.2 ± 1.0	20.8 ± 1.7***	15.8 ± 1.9*	17.40	0.001
MAP (mm Hg)	E_{max}	8.0 ± 1.4	5.1 ± 1.0	25.8 ± 1.9***	16.2 ± 1.9**,††	30.80	0.001
Heart rate (beats/min)	E_{max}	11.3 ± 1.9	12.3 ± 2.4	31.7 ± 3.1***	23.4 ± 3.0**,†	17.90	0.001
Body temperature (°C)	E_{max}	0.41 ± 0.06	0.46 ± 0.07	0.74 ± 0.10*	0.51 ± 0.09	3.40	0.05
Visual analog scales (VAS, %max)							
Any drug effect	E_{max}	1.9 ± 1.3	8.0 ± 3.4	85.4 ± 4.8***	67.7 ± 6.2***,††	120.40	0.001
Drug high	E_{max}	4.9 ± 3.7	9.3 ± 4.6	86.31 ± 4.0***	65.3 ± 8.1***,†	61.10	0.001
Stimulated	E_{max}	2.6 ± 2.6	3.4 ± 2.5	71.9 ± 8.0***	51.1 ± 9.4***,†	34.00	0.001
Closeness	E_{max}	0.25 ± 0.19	0.00 ± 0.00	33.9 ± 5.9***	20.8 ± 4.5***,†	22.70	0.001
Good drug effect	E_{max}	0.06 ± 0.06	6.3 ± 3.4	85.6 ± 4.1***	72.6 ± 7.2***	114.60	0.001
Liking	E_{max}	3.1 ± 3.1	15.6 ± 7.0	86.6 ± 4.7***	77.9 ± 5.1**	75.60	0.001
Adjective Mood Rating Scale (AMRS score)							
Activity	E_{max}	1.56 ± 0.52	1.38 ± 0.45	3.81 ± 0.98*	4.19 ± 1.02**	4.80	0.05
Inactivation	E_{max}	1.88 ± 0.82	3.56 ± 1.03	8.19 ± 2.06	6.50 ± 1.73	4.80	0.05
Extroversion	E_{max}	1.06 ± 0.31	1.25 ± 0.57	4.00 ± 078*	4.13 ± 0.83**	10.10	0.001
Introversion	E_{min}	−0.44 ± 0.20	−1.06 ± 0.35	−3.00 ± 0.82***	−1.19 ± 0.39††	6.20	0.001
Well-being	E_{max}	1.75 ± 0.37	2.56 ± 0.83	6.50 ± 1.28**	6.25 ± 1.46*	16.60	0.001
Emotional excitation	E_{max}	0.44 ± 0.27	1.53 ± 0.69	7.50 ± 1.20***	3.94 ± 1.14*,†	14.20	0.001
Anxiety-depression	E_{max}	0.31 ± 0.18	1.25 ± 0.50	2.25 ± 0.71	1.38 ± 0.62	2.90	0.05
Dreaminess	E_{max}	0.69 ± 0.25	1.63 ± 0.46	4.00 ± 0.74***	3.19 ± 0.68**	7.30	0.001
State-Trait Anxiety Inventory	E_{max}	1.44 ± 0.58	2.19 ± 0.98	10.19 ± 2.36**	3.81 ± 1.06†	9.40	0.001
List of complaints (total score)							
Acute adverse effects	At 3 h	−1.00 ± 0.53	−0.19 ± 1.30	10.13 ± 1.90***	1.94 ± 2.80†	7.73	0.001
Subacute adverse effects	At 24 h	−1.19 ± 0.63	0.06 ± 0.92	5.38 ± 1.47**	0.44 ± 1.33†	5.95	0.01
Ex vivo *binding (K_i, %)*							
NET		24.00 ± 1.28	9.09 ± 1.66***	22.17 ± 1.66	7.16 ± 0.83***,†††	48.02	0.001
SERT		>25	>25	>25	>25		
DAT		>25	>25	>25	>25		

Values are mean ± SEM of changes from baseline of 16 subjects.

DAT, dopamine transporter; DBP, diastolic blood pressure; E_{max}, peak effects; E_{min}, minimum effects; K_i, inhibition constant calculated as % of plasma sample dilution with undiluted plasma set as 100%; MAP, mean arterial pressure; MDMA, 3,4-methylenedioxymethamphetamine; NET, norepinephrine transporter; SBP, systolic blood pressure; SERT, serotonin transporter.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to placebo–placebo. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ compared to placebo–MDMA.

the sympathetic nervous system, as evidenced by increases in heart rate, blood pressure, pupil size, and body temperature.[18] Serious adverse effects of uncontrolled ecstasy use also include hypertensive and hyperthermic reactions that are likely to be mediated by an activation of the adrenergic system by MDMA.[28] The importance of NE in the mechanism of action of amphetamine-type stimulants in general is further supported by the observation that the subjective effects of these stimulants in humans correlate with their potency to release NE and not with their effect on DA.[8] Further support for a role of the NET in the effects of psychostimulants derives from the clinical findings that the NET inhibitor atomoxetine attenuated cocaine-induced systolic blood pressure increases[29] and cardiovascular and subjective responses to D-amphetamine in humans.[30] Together, the preclinical and clinical findings suggest that NE may contribute critically to the psychotropic and, even more importantly, the

cardiovascular effects of MDMA in humans. This study evaluated pharmacokinetic and pharmacodynamic effects of the interaction between the selective NET inhibitor reboxetine and MDMA in healthy subjects. We hypothesized that pretreatment with reboxetine would attenuate the subjective, neuroendocrine, cardiovascular, and adverse effects of MDMA to the extent that they depend on NET-mediated release of NE.

Pharmacokinetic interactions were evaluated to confirm that the effects of reboxetine on the MDMA response could not be explained by the exposure to MDMA or its active metabolites being too low. MDMA is *N*-demethylated to the active, but minor, metabolite 3,4-methylenedioxyamphetamine (MDA) by cytochrome P450 (CYP) 2B6 and 3A4. The major pathway of MDMA degradation includes CYP2D6-mediated O-demethylation to 3,4-dyhydroxymethamphetamine (HHMA), followed by catechol-*O*-methyltransferase-catalyzed methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA).[31]

RESULTS

Pharmacodynamics

MDMA increased the levels of both circulating NE and epinephrine relative to placebo. Reboxetine prevented the MDMA-induced increase in NE, an endocrine correlate of sympathetic activation (Table 1). It also reduced the cardiovascular and psychostimulant effects of MDMA. Reboxetine decreased MDMA-induced elevations in blood pressure and heart rate (Figure 1 and Table 1) and attenuated MDMA-induced visual analog scale (VAS) score increases in "any drug effect," "drug high," "stimulated," and "closeness to others" (Figure 2 and Table 1). In contrast, reboxetine did not affect MDMA-induced VAS score changes with regard to "good drug effect" and "drug liking" (Figure 2 and Table 1). On the 5-Dimensions of Altered States of Consciousness (5D-ASC) Rating Scale, analysis of variance showed significant main effects of the drug in the sum score and in all the dimensions of the scale ($F_{(3,45)}$ = 32.8, 32.9, 8.5, and 17.8 for ASC, oceanic boundlessness (OB), anxious ego dissolution (AED), and visionary restructuralization (VR), respectively; all $P < 0.001$). MDMA robustly increased scores in the OB, AED, and VR dimensions relative to placebo (all $P < 0.001$) (Figure 3). Reboxetine reduced MDMA's effect on the total ASC score ($P < 0.01$) in the OB dimension ($P < 0.01$) and in the VR dimension ($P < 0.05$), including significant reductions in OB item clusters for "experience of unity" ($P < 0.01$) and "blissful state" ($P < 0.1$). With respect to the Adjective Mood Rating Scale (AMRS) scale, reboxetine prevented MDMA-induced increase in emotional excitation and decrease in introversion (Figure 4 and Table 1). MDMA increased a sense of well-being, extroversion, and dreaminess and produced inactivation at 1.25 h and activation at 2 h, which resulted in peak increases in both activity and inactivation relative to placebo. Reboxetine had no effect on these subjective effects associated with MDMA. Finally, reboxetine reduced MDMA-induced elevations in State-Trait Anxiety Inventory (STAI) anxiety scores (Figure 4 and Table 1).

Adverse effects

MDMA increased the total list of complaints adverse effects score at 3 h and again at 24 h after administration, relative to placebo (Table 1). The most frequently reported adverse effects of placebo–MDMA and reboxetine–MDMA included lack of appetite (n = 12 and n = 8, respectively), difficulty in concentrating (n = 12 and n = 12, respectively), tremor (n = 9 and n = 3, respectively), restlessness (n = 8 and n = 4, respectively), and dizziness (n = 6 and n = 4, respectively). Reboxetine decreased the number of MDMA-induced adverse effects (Table 1).

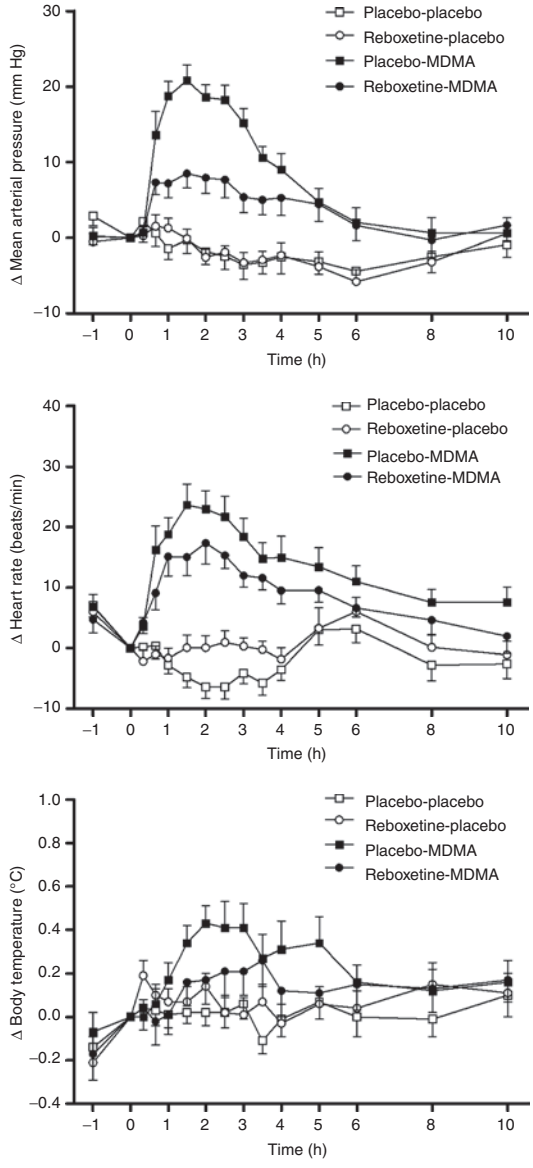

Figure 1 Physiologic effects. Values are mean ± SEM of changes from baseline in 16 subjects. Reboxetine was administered at $t = -12$ h and at $t = -1$ h. 3,4-Methylenedioxymethamphetamine (MDMA) was administered at $t = 0$ h. Reboxetine pretreatment reduced MDMA-induced elevations in blood pressure and heart rate.

Pharmacokinetics

The decrease in the pharmacodynamic response to MDMA after reboxetine pretreatment is not attributable to a pharmacokinetic interaction between reboxetine and MDMA because reboxetine was shown to increase exposure to MDMA. Reboxetine increased the maximum concentration

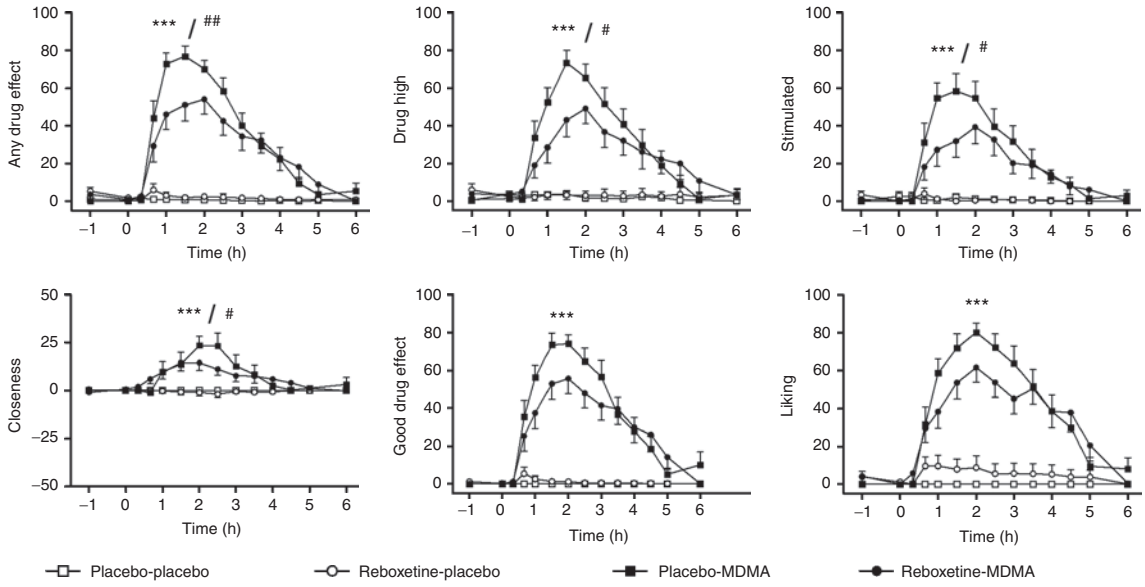

Figure 2 Time courses of subjective visual analog scale (VAS) ratings. Values are mean ± SEM of % maximal values in 16 subjects. Reboxetine decreased 3,4-methylenedioxymethamphetamine (MDMA)-induced elevations in scores for "any drug effect," "drug high," "stimulated," and "closeness to others." $^{***}P < 0.001$ for peak-score differences between placebo–placebo and placebo–MDMA. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for peak-score differences between placebo–MDMA and reboxetine–MDMA.

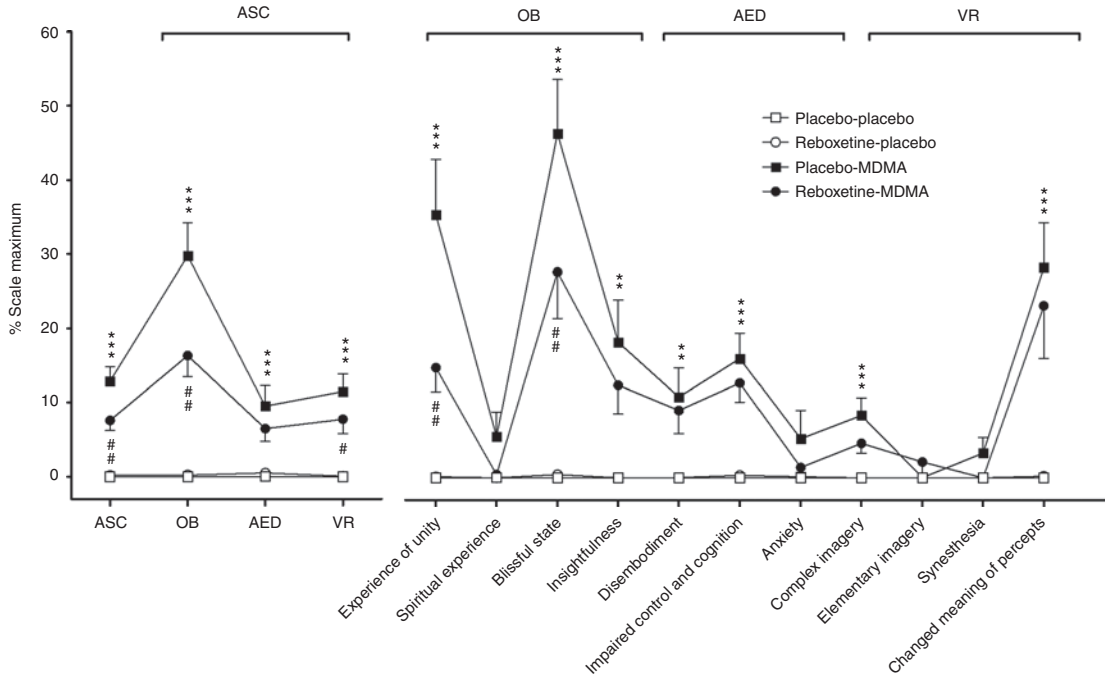

Figure 3 The 5-Dimensions Altered States of Consciousness (5D-ASC) scale. Values are mean ± SEM in 16 subjects. 3,4-Methylenedioxymethamphetamine (MDMA) elicited mainly "experience of unity," "a blissful state," and "changed meaning of percepts." Reboxetine reduced MDMA's effect in the OB dimension, including significant reductions in "experience of unity" and "blissful state." $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to placebo–placebo. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared to placebo–MDMA. AED, anxious ego dissolution; ASC, altered states of consciousness (sum of the scores for OB, AED, and VR); OB, oceanic boundlessness; VR, visionary restructuralization.

(C_{max}) of MDMA by 19 ± 6% ($F_{(1,15)} = 9.23$; $P < 0.01$) and the area under the plasma concentration–time curve $(AUC)_{0-24\,h}$ by 9 ± 4% ($F_{(1,15)} = 5.53$; $P < 0.05$) (**Figure 5a** and **Table 2**). Reboxetine also increased $AUC_{0-24\,h}$ and $AUC_{0-\infty}$ values of MDA by 50 ± 13% ($F_{(1,15)} = 15.98$; $P < 0.001$) and 66 ± 16% ($F_{(1,15)} = 19.03$; $P < 0.001$), respectively (**Figure 5b** and **Table 2**). Conversely, MDMA increased the C_{max} of reboxetine by 16 ± 6% ($F_{(1,15)} = 5.97$; $P < 0.05$) (**Figure 5c** and **Table 2**). The pharmacokinetic parameters of MDMA were not dependent on CYP2D6 phenotype.

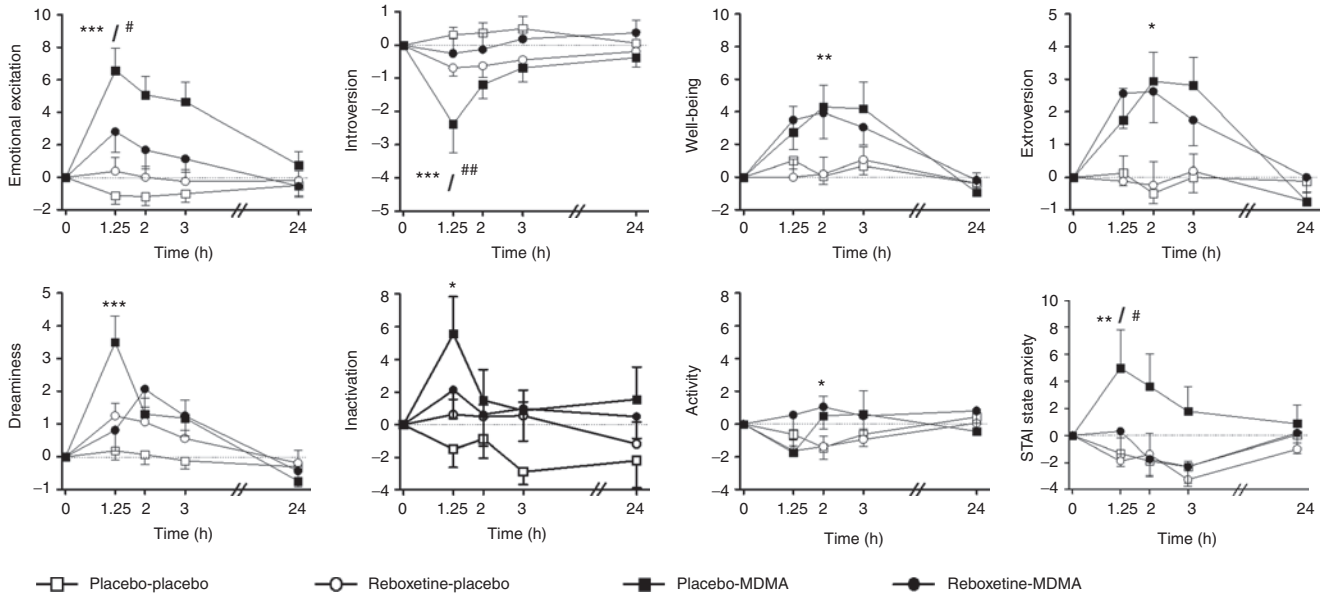

Figure 4 Mood effects in the Adjective Mood Rating Scale (AMRS) and the State-Trait Anxiety Inventory (STAI). Values are mean ± SEM of AMRS/STAI score changes from baseline in 16 subjects. Reboxetine reduced "emotional excitation," and STAI state anxiety produced by 3,4-methylenedioxymethamphetamine (MDMA) and prevented the MDMA-induced decrease in "introversion". $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for peak-score differences between placebo–placebo and placebo–MDMA. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for peak-score differences between placebo–MDMA and reboxetine–MDMA.

Pharmacokinetic–pharmacodynamic relationship

Figure 5d,e shows MDMA's effects in terms of the plasma concentration. MDMA-induced changes in (Figure 5d) mean arterial pressure (MAP) and (Figures 5e) "any drug effect" returned to baseline within 8 h and 6 h, respectively, when MDMA concentrations were still high (clockwise hysteresis). Reboxetine pretreatment attenuated both physical and subjective responses to MDMA (Figure 5d,e).

Ex vivo binding studies

Plasma from subjects treated with reboxetine–placebo or reboxetine–MDMA inhibited *ex vivo* radioligand binding to NET but not to SERT or DAT (Table 1).

DISCUSSION

In this study, pretreatment with the selective NET inhibitor reboxetine prevented MDMA-induced increase in circulating levels of NE, which is a marker of sympathetic system activation, and significantly reduced the cardiovascular response to MDMA. Reboxetine also attenuated some, but not all, of the psychotropic effects of MDMA and reduced MDMA-induced drug high, stimulation, emotional excitation, and anxiety, as well as the blissful state and experience of unity elicited by MDMA. Reboxetine also ameliorated some of the adverse effects of MDMA such as tremor and restlessness. Overall, blockade of NET resulted in a pronounced decrease in the cardiovascular stimulant effects of MDMA and a moderate attenuation of its psychostimulant properties. In contrast, good drug effects and the sense of well-being associated with MDMA were not significantly altered by reboxetine pretreatment. The findings are consistent with a role for NET in the mediation of the sympathomimetic stimulant-like aspects of the MDMA effect.

The pharmacodynamic interaction observed in this study cannot be explained on the basis of a pharmacokinetic interaction between reboxetine and MDMA because reboxetine decreased the pharmacodynamic effects of MDMA even while it increased the C_{max} of MDMA by 20% and the $AUC_{0-24\,h}$ of MDMA and its active metabolite MDA by 10% and 50%, respectively. The potent CYP2D6 inhibitor paroxetine has previously been shown to increase the C_{max} and $AUC_{0-27\,h}$ of MDMA by 20% and 30%, respectively, and of MDA by 20% and 20%, respectively[31] (similar to the effect of reboxetine in our study), whereas it decreased MDMA metabolism to HHMA and HMMA.[31] The effects of paroxetine and reboxetine on MDMA metabolism can therefore be explained by CYP2D6 inhibition[32] and a shift of the metabolism from the major pathway (by reducing HHMA and HMMA formation) to a minor pathway (including an increase in MDA formation). However, we did not measure HHMA and HMMA levels in plasma, and this may be a limitation with respect to the conclusion regarding pathways. Furthermore, MDMA is itself a mechanism-based inhibitor of CYP2D6, and its pharmacokinetics is nonlinear.[33,34] In this study, we used an *ex vivo* binding assay to investigate whether the plasma samples taken from the subjects exhibited NET-binding properties. We confirmed that plasma from reboxetine-treated subjects displaced ^{3}H-nisoxetine from NET and that there was a trend toward this effect with regard to plasma from MDMA-treated subjects. Although both reboxetine and MDMA bind to NET *in vitro* and inhibit NE uptake,[6,35] MDMA is also a NET substrate and releases NE.[6] The pharmacodynamic interaction between reboxetine and MDMA observed in this study is consistent with

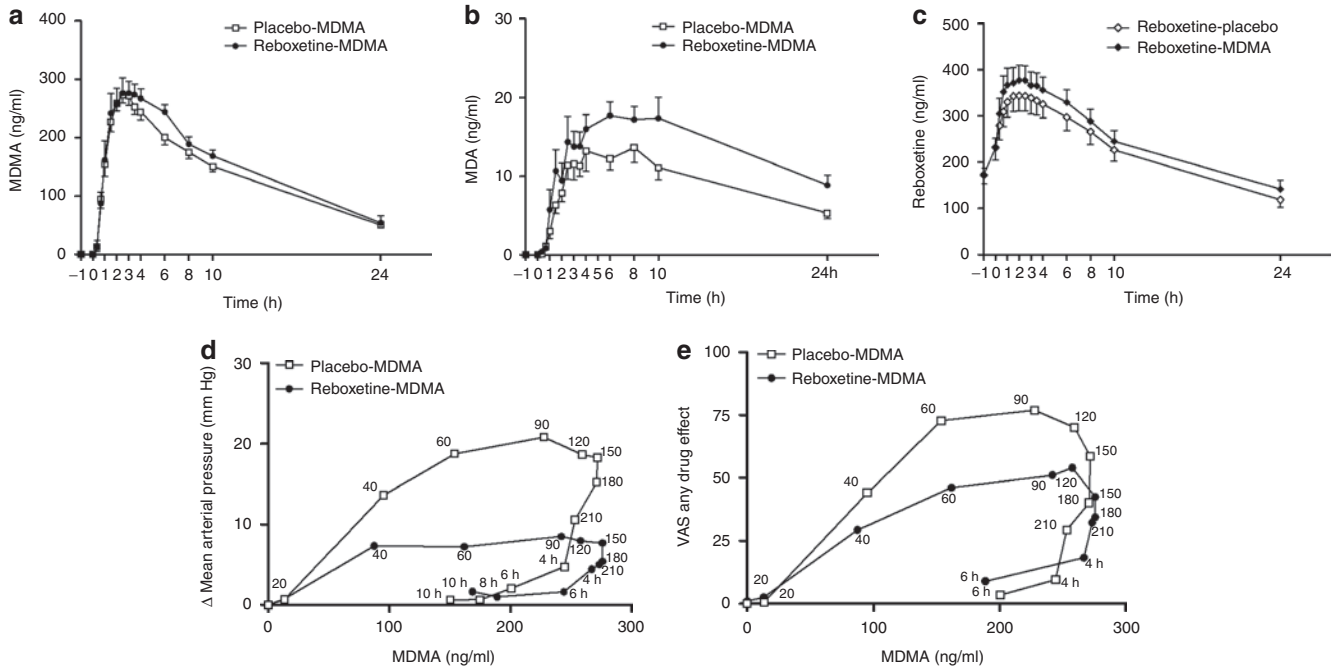

Figure 5 Pharmacokinetics of (**a**) MDMA, (**b**) MDA, and (**c**) reboxetine. The values are mean ± SEM in 16 subjects. Reboxetine was administered at $t = -12$ h and at $t = -1$ h. MDMA was administered at $t = 0$ h. Reboxetine increased the exposure to (**a**) MDMA (C_{max} and $AUC_{0-24\,h}$) and (**b**) MDA ($AUC_{0-24\,h}$). Conversely, (**c**) MDMA increased the C_{max} of reboxetine. (**d**,**e**) MDMA effects plotted against MDMA plasma concentrations. The values are the means of the change from baseline in 16 subjects, with SEM omitted for clarity. The time of sampling is noted next to each point in minutes or hours after MDMA administration. Less effect is seen at the same MDMA concentration at later time points. MDMA-induced changes in mean arterial pressure and "any drug effect" VAS score returned to baseline within 8 h and 6 h, respectively, even when MDMA concentrations remained high. This clockwise hysteresis indicates acute tolerance to the MDMA effect, possibly because of monoamine depletion, resulting in no effect until monoamine stores are refilled. Reboxetine pretreatment lowered both physical and subjective responses to MDMA. $AUC_{0-24\,h}$, area under the plasma concentration–time curve from 0 to 24 h; C_{max}, maximum plasma concentration; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; VAS, visual analog scale.

Table 2 Pharmacokinetic parameters of MDMA, MDA, and reboxetine

	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0-24\,h}$ (ng/ml·h)	$AUC_{0-\infty}$ (ng/ml·h)
MDMA					
Placebo–MDMA	298.0 ± 12.3	2.6 ± 0.2	9.6 ± 0.8	3,357.1 ± 159.0	4,127.2 ± 229.2
Reboxetine–MDMA	351.0 ± 19.0**	2.8 ± 0.4	7.0 ± 0.3	3,629.1 ± 167.5*	4,106.2 ± 215.8
MDA					
Placebo–MDA	19.0 ± 2.1	4.7 ± 0.6	16.1 ± 1.8	227.4 ± 19.0	358.6 ± 25.0
Reboxetine–MDA	23.0 ± 2.7	7.0 ± 0.6	17.3 ± 1.9	327.7 ± 24.0**	572.2 ± 48.9***
Reboxetine					
Reboxetine–placebo	371.7 ± 33.9	3.3 ± 0.4	13.7 ± 0.7	5,704.4±599.6	8,292.5 ± 1,042.2
Reboxetine–MDMA	416.9 ± 31.1†	3.6 ± 0.5	13.2 ± 0.9	6,176.2 ± 553.2	9,103.4 ± 1,099.9

Values are mean ± SEM of 16 healthy subjects.

AUC, area under the plasma concentration–time curve; C_{max}, maximum plasma concentration; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; $T_{1/2}$, terminal elimination half-life; T_{max}, time to maximum plasma concentration.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to placebo–MDMA. †$P < 0.05$ compared to reboxetine–placebo.

inhibition of the MDMA-induced NET-mediated NE release by reboxetine.

The role for NET in the mechanism of action of MDMA, as suggested by the results of this study, is in line with both preclinical and clinical data from other studies, as outlined in the introduction. As compared to SERT and DAT, MDMA shows higher affinity to NET *in vitro*[5,6] and releases NE more potently than 5-HT or DA.[6,8] In humans, NET inhibition reduced cardiostimulant responses to cocaine[29] and both cardiostimulant and psychostimulant responses to D-amphetamine,[30] similar to the findings in our study with MDMA.

The SERT inhibitor citalopram has been shown to reduce the cardiovascular and subjective effects of MDMA in humans.[18,19] Citalopram reduced MDMA-induced increases in systolic blood pressure and emotional excitation by <50% and positive mood effects by >50%. In contrast, reboxetine reduced MDMA-

induced increases in systolic blood pressure and emotional excitation by >50% and positive mood effects by <50%. The two studies indicate that SERT-mediated 5-HT release is more important than NET-mediated NE release for MDMA-typical positive mood effects, whereas NET-mediated NE release primarily mediates the more stimulant-typical emotional excitation and cardiovascular response to MDMA. The effect of reboxetine on subjective responses to MDMA is similar to changes in the subjective effects of D-amphetamine after pretreatment with the NET inhibitor atomoxetine.[30] As in our study, NET inhibition reduced amphetamine-induced increases in subjective ratings of "stimulated" and "high" but not in "drug liking,"[30] thereby reinforcing the view that NET contributes mainly to the psychostimulant aspect of amphetamines.[36]

DA is commonly thought to mediate the reinforcing and rewarding effects of drugs of abuse. For example, the DAT/NET inhibitor methylphenidate has been shown to reduce intravenous amphetamine use in amphetamine-dependent patients.[37] The role of DA in the mediation of the acute subjective effects of amphetamine-type stimulants in humans is less clear. The DAT/NET inhibitor bupropion was shown to attenuate subjective responses to methamphetamine.[38] However, the effect of DAT inhibition on the acute response to MDMA has not been studied in humans. We have previously shown that the DA D_2 antagonist haloperidol reduces the positive mood elicited by MDMA and that haloperidol depresses mood also when given alone, as compared to the effect of placebo.[15] Similarly, DA D_2 receptor blockade did not affect subjective responses to D-amphetamine, according to the results of most studies.[13,14] Accordingly, DA may primarily mediate the reinforcing properties of psychostimulants but might not be the primary mediator of their acute effects.[8]

The exact mechanism by which monoamine transport inhibitors interact with MDMA-induced monoamine release is not known. The SERT, DAT, and NET inhibitor indatraline blocks MDMA-induced transmitter release according to simple competitive models.[7] Other inhibitors alter the efficiency of the MDMA-induced transmitter release in a noncompetitive manner, possibly by inducing conformational changes in the transporter protein.[7] A channel-like conformation of DAT, resulting in rapid DA efflux, has also been described for amphetamine-induced DA release.[39]

The present study adds to a better understanding of the mechanism of action of MDMA. Our finding that reboxetine reduces the subjective effects of MDMA (stimulant and drug high) is similar to the finding from another study that atomoxetine attenuates the subjective effects of D-amphetamine.[30] Taken together, these findings indicate that NET inhibitors may potentially be useful as treatments for stimulant addiction.[36,40] However, further clinical studies are needed to explore the therapeutic potential of NET inhibitors in stimulant dependence.

In summary, we showed that NE plays a critical role in the acute physiologic and subjective effects of MDMA in humans.

METHODS

Study design. We used a double-blind, placebo-controlled, randomized, crossover design with four experimental conditions (placebo–placebo, reboxetine–placebo, placebo–MDMA, and reboxetine–MDMA). The order of the four test sessions was counterbalanced. Washout periods between sessions were 10–14 days long. The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Guidelines on Good Clinical Practice and was approved by the Ethics Committee of the Canton of Basel, Switzerland. The use of MDMA in healthy subjects was authorized by the Swiss Federal Office of Public Health, Bern, Switzerland. The study was registered at ClinicalTrials.gov (NCT00886886).

Study outline. Subjects completed a screening session, four test sessions with a next-day follow-up, and an end-of-study visit. Test sessions took place in a quiet hospital research ward with no more than two research subjects present per session. Prior to admission to the test sessions, the subjects were asked about potential health problems; drug tests and urine tests for pregnancy were also performed. An indwelling intravenous catheter was placed in the antecubital vein for blood sampling. Reboxetine (8 mg orally) or placebo was administered at 20:00 h the day before the test session and again at 7:00 h after a light meal on the day of the test. MDMA (125 mg orally) or placebo was administered at 8:00 h, 1 and 12 h after reboxetine. A standardized lunch was served at 12:00 h, and subjects were sent home at 18:00 h. On the day following each test session, the subjects returned to the research ward at 8:00 h for the assessment of adverse effects and blood sampling. During the test sessions, the subjects did not drink beverages containing caffeine or alcohol. They were reading, listening to music, or walking around in the research ward. For most of the time, they were sitting or lying comfortably. Outcome measures were assessed repeatedly before and after drug administration.

Subjects. Sixteen healthy subjects (eight men and eight women), 20–44 years of age (mean ± SD: 25.7 ± 5.5 years), were recruited on the university campus by word of mouth. Exclusion criteria included: age <18 or >45 years, pregnancy (urine pregnancy test before each test session), abnormal body mass index (<18.5 or >25 kg/m^2), personal or family (first-degree relative) history of psychiatric disorder (as assessed by the structured clinical interview for axis I and II disorders according to the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV),[41] supplemented by psychometric instruments),[42] regular use of medications, chronic or acute physical illness (as assessed by physical examination, electrocardiogram, standard hematological, and chemical blood analyses), smoking (more than 10 cigarettes/day), lifetime history of illicit drug use more than five times (except for tetrahydrocannabinol), illicit drug use within the past 2 months, and illicit drug use during the study (urine tests for drug use before test sessions using TRIAGE 8, Biosite, San Diego, CA). The subjects were asked to abstain from excessive alcohol consumption between test sessions and, in particular, to limit alcohol use to one glass on the day before each test session. Three subjects were light smokers (fewer than 10 cigarettes/day). They maintained their usual smoking habit but were not allowed to smoke for 6 h after MDMA/placebo administration. Eleven subjects had previously used cannabis. Six subjects had illicit drug experiences (one to four times): one subject had tried cocaine, one had tried ecstasy, two had tried psilocybin, one had tried psilocybin and ecstasy, and one had tried ecstasy, psilocybin, and cocaine. The three subjects with ecstasy experience had all used the drug only once. All the subjects were phenotyped for CYP2D6 activity, using dextrometorphan as the probe drug. There were 10 extensive, 4 intermediate, and 2 poor CYP2D6 metabolizers in the study. All subjects gave their written informed consent before participating in the study, and they were paid for their participation.

Study drugs. (±) MDMA hydrochloride (Lipomed AG, Arlesheim, Switzerland) was obtained from the Swiss Federal Office of Public Health and prepared as gelatin capsules (100 mg and 25 mg) by Bichsel Laboratories AG, Interlaken, Switzerland, in accordance with good manufacturing practice. Identical placebo (lactose) capsules were prepared. MDMA was administered in a single absolute dose of 125 mg, corresponding to a dose of 1.85 ± 0.24 mg/kg body weight. This dose

of MDMA corresponds to a typical recreational dose of ecstasy, and comparable doses of MDMA have previously been used in controlled settings.[2–4,43] Reboxetine is a potent, selective, and specific NE uptake inhibitor.[35] Reboxetine (8 mg, Edronax; Pfizer, Zurich, Switzerland) and identical-looking placebo (lactose) capsules were similarly prepared by Bichsel Laboratories. Reboxetine (8 mg) or placebo was administered twice, 12 h and 1 h before MDMA (125 mg) or placebo. Similar dosing regimens have previously been used to manipulate the NE system function in healthy subjects.[44]

Pharmacodynamics

Psychometric scales: Subjective measures included VAS,[21] the AMRS,[45] the 5D-ASC,[46] and the STAI.[42]

VAS: VASs included "any drug effect," "drug high," "stimulated," "closeness to others," "good drug effect," and "liking."[3,20,21] VASs were presented as 100-mm horizontal lines marked "not at all" on the left and "extremely" on the right. The VAS for "closeness to others" was bidirectional (± 50 mm). VAS tests were administered 1 h before and at 0, 0.33, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 h after MDMA/placebo administration.

5D-ASC: The 5D-ASC rating scale measures alterations in mood, perception, experience of self in relation to environment, and thought disorder. The instrument comprises five subscales (dimensions)[46] and eleven lower-order scales[47]: The 5D-ASC dimension OB (27 items) measures derealization and depersonalization associated with positive emotional states ranging from heightened mood to euphoric exaltation. The corresponding lower-order scales are "experience of unity," "spiritual experience," "blissful state," and "insightfulness." The dimension AED (21 items) summarizes ego disintegration and loss of self-control, phenomena associated with anxiety. The corresponding lower-order scales are "disembodiment," "impaired control of cognition," and "anxiety." The dimension "VR (18 items) consists of the lower-order scales "complex imagery," "elementary imagery," "audiovisual synesthesia," and "changed meaning of percepts." Two other dimensions of the scale were not used in our study. The global ASC score was constructed by adding the OB, AED, and VR scores. The 5D-ASC scale was administered 4 h after administration of MDMA or placebo.

AMRS: The 60-item Likert-scale short version of the AMRS[45] was administered 1 h before and at 1.25, 2, 3, and 24 h after MDMA or placebo. The AMRS contains subscales for activity, inactivation, extroversion and introversion, well-being, emotional excitation, anxiety–depression, and dreaminess.

STAI: The STAI state-anxiety scale[42] test was administered 1 h before and at 1.25, 2, and 3 h after MDMA or placebo.

Physiologic measures. Physiologic measures were assessed repeatedly, at −1, 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, and 10 h after administration of MDMA or placebo. Heart rate, systolic blood pressure, and diastolic blood pressure were measured using an OMRON M7 blood pressure monitor (OMRON Healthcare Europe, Hoofddorp, The Netherlands) in the dominant arm after a resting time of 5–10 min, with the volunteer sitting in bed with the back supported. Measures were taken twice per time point with an interval of 1 min, and the average was used for analysis. Between measurements, subjects were allowed to engage in nonstrenuous activities. Core (tympanic) temperature was assessed using a GENIUS 2 ear thermometer (Tyco Healthcare Group, Watertown, NY). The temperature of the room was maintained at 22.5 ± 0.5 °C.

Adverse effects. Adverse effects were assessed at 0, 3, and 24 h after administration of MDMA or placebo by using the List of Complaints.[2,48] The scale consists of 66 items, yielding a total adverse effects score (nonweighted sum of the item answers), reliably measuring physical and general discomfort. The scale has previously been shown to be sensitive to the adverse effects of MDMA.[2,22]

Blood collection for endocrine and pharmacokinetic measurements. Samples of whole blood for the determination of MDMA, MDA, and reboxetine levels were collected into lithium heparin monovettes at −1, 0, 0.33, 0.66, 1, 1.5, 2,3.5, 3, 3.5, 4, 6, 8, 10, and 24 h after administration of MDMA or placebo. Blood samples to determine concentrations of NE and epinephrine were taken 60 min after administration of MDMA or placebo. All blood samples were collected on ice and centrifuged within 10 min at 4 °C. Plasma was then stored at −70 °C until analysis.

Laboratory analyses

Catecholamines: The levels of free catecholamines (NE and epinephrine) were determined using a modified method of the RECIPE kit (ClinRep; RECIPE Chemicals and Instruments, Munich, Germany) (see **Supplementary Methods** online). The lower limit of quantification was 20 pmol/l, and interassay precisions (coefficient of variation (CV)) were <15%.

MDMA and MDA: Plasma concentrations of MDMA and its active metabolite, 3,4-methylenedioxyamphetamine (MDA), were determined using high-performance liquid chromatography with diode-array detection[49] (see **Supplementary Methods** online). The limit of quantification was 5 ng/ml for MDMA and 2 ng/ml for MDA. Interday precision values (CV) were 7 and 4%, and interday accuracy values were 96–106% and 100–103% for MDMA and MDA, respectively.

Reboxetine: Plasma reboxetine concentrations were analyzed using liquid chromatography–mass spectrometry (see **Supplementary Methods** online). The limit of quantification was 34.5 ng/ml. Interday precision (CV) values were 5.7 and 3.2%, and interday accuracy values were 98.5 and 101.8%, at 92 ng/ml and at 344 ng/ml, respectively.

Ex vivo *binding*: Plasma samples for investigating *ex vivo* binding were collected 60 min after administration of MDMA or placebo. We determined the potencies of plasma to inhibit ^{3}H-nisoxetine, ^{3}H-citalopram, and ^{3}H-WIN35, 428 binding to NET, SERT, and DAT, respectively (see **Supplementary Methods** online). K_i values were calculated as percentages of plasma sample dilutions required for obtaining 50% of maximum effect (10 μmol/l indatraline in human plasma was used to achieve 100% inhibition). Undiluted plasma samples were set as 100%. Therefore, a K_i of 10% indicates that a plasma sample diluted 10-fold displaced 50% of the radioligand.

Pharmacokinetics. Data for plasma concentrations of MDMA, MDA, and reboxetine were analyzed using noncompartmental methods (WinNonlin; Pharsight, Mountain View, CA). C_{max} and time to maximum concentration (T_{max}) were obtained directly from the concentration–time curves of observed values. 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. Terminal elimination half-life ($t_{1/2}$) was calculated using λ_z and the equation $t_{1/2} = \ln_2/\lambda_z$. The $AUC_{0-24\,h}$ was calculated using the linear trapezoidal rule. The $AUC_{0-\infty}$ was determined by extrapolation of the $AUC_{0-24\,h}$, using λ_z.

Statistical analysis. Values were transformed to differences from baseline. Peak effects (E_{max}) were determined for repeated measures. E_{max} values were compared by one-way General Linear Models repeated measures analysis of variance with drug as a factor, using STATISTICA 6.0 (StatSoft, Tulsa, OK). Tukey *post hoc* comparisons were performed based on significant main effects of treatment. Additional analyses of variance were performed with drug order as an additional factor so as to exclude carryover effects. The criterion for significance was $P < 0.05$. MAP was calculated from diastolic blood pressure and systolic blood pressure using the formula MAP = DBP + (SBP−DBP)/3.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Paper 2b

Duloxetine Inhibits Effects of MDMA ("Ecstasy") In Vitro and in Humans in a Randomized Placebo-Controlled Laboratory Study

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Duloxetine Inhibits Effects of MDMA (''Ecstasy'') *In Vitro* and in Humans in a Randomized Placebo-Controlled Laboratory Study

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Abstract

This study assessed the effects of the serotonin (5-HT) and norepinephrine (NE) transporter inhibitor duloxetine on the effects of 3,4–methylenedioxy-methamphetamine (MDMA, ecstasy) *in vitro* and in 16 healthy subjects. The clinical study used a double-blind, randomized, placebo-controlled, four-session, crossover design. *In vitro,* duloxetine blocked the release of both 5-HT and NE by MDMA or by its metabolite 3,4-methylenedioxyamphetamine from transmitter-loaded human cells expressing the 5-HT or NE transporter. In humans, duloxetine inhibited the effects of MDMA including elevations in circulating NE, increases in blood pressure and heart rate, and the subjective drug effects. Duloxetine inhibited the pharmacodynamic response to MDMA despite an increase in duloxetine-associated elevations in plasma MDMA levels. The findings confirm the important role of MDMA-induced 5-HT and NE release in the psychotropic effects of MDMA. Duloxetine may be useful in the treatment of psychostimulant dependence.

Trial Registration: Clinicaltrials.gov NCT00990067

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☯ These authors contributed equally to this work.

Introduction

Amphetamine derivatives, including 3,4-methylenedioxy-methamphetamine (MDMA, ''ecstasy'') bind to monoamine transporters and potently release serotonin (5-hydroxytryptamine [5-HT]), norepinephrine (NE), and dopamine (DA) through the 5-HT (SERT), NE (NET), and DA (DAT) transporters, respectively [1,2,3,4]. The pharmacological effect of MDMA can be blocked by monoamine transporter inhibitors. *In vitro*, the MDMA-induced release of NE, DA, or 5-HT from rat brain synaptosomes preloaded with monoamines is competitively inhibited by the monoamine transporter inhibitor indatraline [5,6]. In humans, SERT inhibition reduced the psychotropic response to MDMA [7,8,9]. NET inhibition also attenuated the acute effects of MDMA [10] and amphetamine [11] in humans. In contrast, clonidine, which inhibits the vesicular release of NE, did not inhibit the effects of MDMA in humans [12]. Thus, the available evidence indicates that the MDMA-induced transporter-mediated release of 5-HT and NE appears to be involved in aspects of the acute subjective and cardiovascular responses to psychostimulants [2,7,10,11]. However, the response to MDMA in humans was only moderately affected when either the SERT or NET was pharmacologically blocked [7,10]. Therefore, we evaluated the effects of dual SERT and NET inhibition with duloxetine on the pharmacokinetics (PK) and pharmacodynamics (PD) of MDMA in humans. Duloxetine was used because it is the most potent and selective dual SERT and NET inhibitor, although it also inhibits the DAT with 10- to 100-fold lower potency compared with the SERT and NET [13,14]. MDMA is mainly metabolized to 3,4-dihydroxymethamphetamine (HHMA) by cytochrome P450 (CYP) 2D6-mediated *O*-demethylation, followed by catechol-*O*-methyltransferase-catalyzed methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA) [15]. Because duloxetine inhibits CYP 2D6 [16], we expected an increase in plasma MDMA concentrations after duloxetine pretreatment. MDMA is also *N*-demethylated to the active metabolite 3,4-mehthylenedioxyamphetamine (MDA). Whether the effects of MDA on 5-HT and NE release are inhibited by transporter inhibitors is unknown. Additionally, the inhibition of MDMA's effect on 5-HT and NE release by duloxetine has not been studied. Therefore, we also assessed the effects of duloxetine on 5-HT and NE release induced by MDMA or MDA *in vitro* using cells that express the respective human transporters. We also sought to link the *in vitro* and *in vivo* data to provide additional insights into the differential modulatory

role of 5-HT and NE in the effects of MDMA in humans. Because the data on monoamine transporter affinity and inhibition have mostly been derived from studies that used rat transporters [17], we investigated the binding and inhibition characteristics of the human monoamine transporters for MDMA, MDA, and duloxetine and the transporter inhibitors used in previous clinical studies [7,8,9,10] and *in vitro* studies [5,6]. Finally, we used an *ex vivo* binding assay to assess whether plasma samples taken from the drug-treated participants in the clinical study exhibit SERT, NET, and DAT-binding properties *ex vivo*.

The overall hypothesis of the present study was that duloxetine would potently bind to SERT and NET and block the MDMA- and MDA-induced transporter-mediated release of 5-HT and NE *in vitro* and markedly reduce the acute effects of MDMA *in vivo* in humans.

Methods

Clinical Study

The protocol for the clinical trial, the CONSORT checklist, and the CONSORT flowchart are available as supporting information; see Protocol S1, Checklist S1, and Figure 1. There were no changes to the protocol during the study.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton of Basel, Switzerland. All of the subjects provided written informed consent before participating in the study, and they were paid for their participation.

Design

We used a double-blind, placebo-controlled, randomized, crossover design with four experiential conditions (placebo-placebo, duloxetine-placebo, placebo-MDMA, and duloxetine-MDMA) in a balanced order. The washout periods between the sessions were at least 10 days long.

Participants

Sixteen healthy subjects (eight men, eight women) with a mean±SD age of 26.1±6.0 years participated in the study. The allocation to treatment order was performed by drawing from blocks of eight different balanced drug treatment sequences by a pharmacist not involved in the study. Each code was stored in a sealed envelope until the termination of the study. Data from all 16 subjects were available for the final analysis (Figure 1). The sample-size estimation showed that 13 subjects would be needed to detect a meaningful reduction of 20% of the MDMA drug effect by duloxetine with more than 80% power using a within-subjects study design. The exclusion criteria included the following; *(i)* age <18 or >45 years, *(ii)* pregnancy determined by a urine test before each session, *(iii)* body mass index <18.5 kg/m^2 or >25 kg/m^2, *(iv)* personal or family (first-degree relative) history of psychiatric disorder (determined by the structured clinical interview of Axis I and Axis II disorders according the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition [18] supplemented by the SCL-90-R Symptom Checklist [19,20] *(v)* regular use of medications, *(vi)* chronic or acute physical illness assessed by physical examination, electrocardiogram, standard hematological, and chemical blood analyses, *(vii)* smoking more than 10 cigarettes per day, *(viii)* a lifetime history of using illicit drugs more than five times with the exception of cannabis, *(ix)* illicit drug use within the last 2 months, and *(x)* illicit drug use during the study determined by urine tests conducted before the test sessions. None of the 16 subjects had used ecstasy previously. The subjects were asked to abstain from excessive alcohol consumption between the test sessions and limit their alcohol use to one glass on the day before the test session. All of the subjects were phenotyped for cytochrome P450 (CYP) 2D6

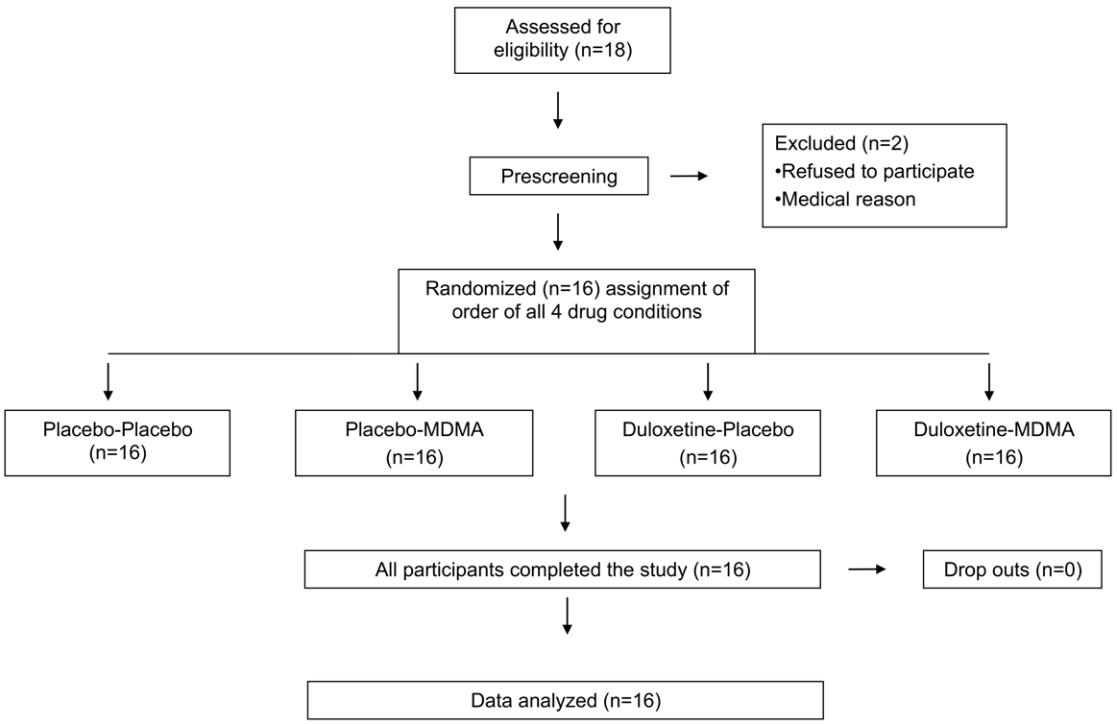

Figure 1. CONSORT flowchart.
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activity using dextromethorphan. Thirteen extensive, two intermediate, and one poor CYP 2D6 metabolizer were identified in the study. The female subjects were investigated during the follicular phase (day 2–14) of their menstrual cycle.

Drugs

(±)MDMA hydrochloride ($C_{11}H_{15}NO_2$, Lipomed, Arlesheim, Switzerland) was obtained from the Swiss Federal Office of Public Health and prepared as gelatin capsules (100 mg and 25 mg). Identical placebo (lactose) capsules were prepared. MDMA was administered in a single absolute dose of 125 mg that corresponded to an average dose of 1.87±0.36 mg/kg body weight. This dose of MDMA corresponds to a typical recreational dose of ecstasy, and comparable doses of MDMA have previously been used in controlled settings. Duloxetine (Cymbalta, Eli Lilly, Vernier, Switzerland) was prepared as 60 mg gelatine capsules, and identically looking placebo (lactose) capsules were similarly prepared. Duloxetine (120 mg) or placebo was administered twice 16 and 4 h before MDMA or placebo administration, respectively. The dose of the two administrations of duloxetine (120 mg/day on two separate days) was in the upper range of the chronic doses used clinically (60–120 mg/day). This dosing schedule was used to obtain high plasma concentrations of duloxetine similar to those reached with chronic administration of 60 mg/day. Drugs were administered without food.

Assessments

Psychometric measures. The psychometric measures included Visual Analog Scales (VAS) [8,10], the Adjective Mood Rating Scale (AMRS) [21], and 5-Dimensions of Altered States of Consciousness (5D-ASC) [22,23]. The VASs included "any drug effect," "good drug effect," "bad drug effect," "drug liking," "drug high," "stimulated," "fear," "closeness to others," "talkative," and "open" [8,10,12,24,25]. The VASs were presented as 100 mm horizontal lines marked "not at all" on the left and "extremely" on the right. The VASs for "closeness to others," "open," and "talkative" were bidirectional (±50 mm). The VASs were administered 4 h before and 0, 0.33, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 5 h after MDMA or placebo administration. The 60-item Likert-type scale of the short version of the AMRS [21] was administered 4 h before and 1.25, 2, and 5 h after MDMA or placebo administration. The AMRS contains subscales for activity, extroversion and introversion, well-being, emotional excitation, anxiety-depression, and dreaminess. The 5D-ASC rating scale measures alterations in mood, perception, experience of self in relation to the environment, and thought disorder. The 5D-ASC rating scale comprises five subscales or dimensions [22] and 11 lower-order scales [23]. The 5D-ASC dimension "oceanic boundlessness" (OB, 27 items) measures derealization and depersonalization associated with positive emotional states, ranging from heightened mood to euphoric exaltation. The corresponding lower-order scales include "experience of unity," "spiritual experience," "blissful state," and "insightfulness." The 5D-ASC dimension "anxious ego dissolution" (AED, 21 items) summarizes ego disintegration and loss of self-control phenomena, two phenomena associated with anxiety. The corresponding lower-order scales include "disembodiment," "impaired control of cognition," and "anxiety." The dimension "visionary restructuralization" (VR, 18 items) consists of the lower-order scales "complex imagery," "elementary imagery," "audiovisual synesthesia," and "changed meaning of percepts." Two other dimensions of the scale were not used in our study. The global ASC score was determined by adding the OB, AED, and VR scores. The 5D-ASC scale was administered 4 h after MDMA or placebo administration.

Physiologic measures. Physiologic measures were assessed repeatedly 4, 3, 2, and 1 h before and 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after MDMA or placebo administration. Heart rate, systolic blood pressure, and diastolic blood pressure were measured using an OMRON M7 blood pressure monitor (OMRON Healthcare Europe, Hoofddorp, The Netherlands). Measures were taken twice per time point with an interval of 1 min, and the average was used for the analysis. Core (tympanic) temperature was assessed using a GENIUS 2 ear thermometer (Tyco Healthcare Group, Watertown, NY). The temperature of the room was maintained at 23.2±0.5°C. Adverse effects were assessed using the List of Complaints (LC) [26], which consists of 66 items that yield a total adverse effects score and reliably measure physical and general discomfort.

Plasma catecholamines and Pharmacokinetics (PK). Blood samples to determine the concentrations of NE and epinephrine were collected 4 h before and 1 and 2 h after MDMA or placebo administration. The levels of free catecholamines (NE and epinephrine) were determined using high-performance liquid chromatography (HPLC) with an electrochemical detector as described previously [10]. Plasma concentrations of copeptin were also determined in this study as reported elsewhere [27]. Samples of whole blood for the determination of MDMA, MDA, HMMA, and duloxetine were collected into lithium heparin monovettes -4, 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, and 6 h after administration of MDMA or placebo. Plasma concentrations of MDMA, MDA, HMMA, and duloxetine were analyzed by HPLC coupled to a tandem mass spectrometer as described previously [12]. The assays were linear in the concentration ranges of 1–1000 ng/ml for MDMA and MDA, 1–500 ng/ml for HMMA, and 2.5–1000 ng/ml for duloxetine. The performance of the method was monitored using quality control (QC) samples at the lower limit of quantification (LLOQ) and at two or three QC concentrations. The interassay accuracy values for the QC samples ranged from 97.5% to 100% for MDMA, from 95.3% to 103% for MDA, from 91.1% to 106% for HMMA, and from 93.2% to 96.4% for duloxetine. The interassay precision values ranged from 2.8% to 8.0% for MDMA, from 3.8% to 10.5% for MDA, from 3.1% to 8.8% for HMMA, and from 4.7% to 9.3% for duloxetine. No hydrolysis was performed. Thus, the values for HMMA represent the drug concentrations of the non-conjugated metabolite. All blood samples were collected on ice and centrifuged within 10 min at 4°C. The plasma was then stored at –20°C until the analysis.

In vitro Studies

Binding to monoamine transporters in vitro. Human embryonic kidney (HEK) 293 cells (Invitrogen, Zug, Switzerland) stably transfected with the human NET, SERT, or DAT as previously described [28] were cultured. The cells were collected and washed three times with phosphate-buffered saline (PBS). The pellets were frozen at –80°C. The pellets were then resuspended in 400 ml of 20 mM HEPES-NaOH, pH 7.4, that contained 10 mM EDTA at 4°C. After homogenization with a Polytron (Kinematica, Lucerne, Switzerland) at 10000 rotations per minute (rpm) for 15 s, the homogenates were centrifuged at 48000× g for 30 min at 4°C. Aliquots of the membrane stocks were frozen at –80°C. All assays were performed at least three times. The test compounds were diluted in 20 µl of binding buffer (252 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 3.52 mM KH_2PO_4, pH 7.4) and 10 point dilution curves were made and transferred to 96-well white polystyrene assay plates (Sigma-Aldrich, Buchs, Switzerland). *N*-

methyl-^{3}H-nisoxetine (~87 C_i/mmol, Perkin-Elmer) was the radioligand for the NET assay and had a dissociation constant (K_d) of 9 nM. Fifty microliters of 12 nM [^{3}H]-nisoxetine was added to each well of the assay plates, targeting a final [^{3}H]-nisoxetine concentration of 3 nM. [^{3}H]-citalopram (~72 C_i/mmol; Perkin-Elmer) was the radioligand for the SERT assay and had a K_d of 2.2 nM. Fifty microliters of 8 nM [^{3}H]-citalopram was added to each well of the SERT assay plates, targeting a final [^{3}H]-citalopram concentration of 2 nM. [^{3}H]-WIN35,428 (~86 C_i/mmol; Perkin-Elmer) was the radioligand for the DAT assay and had a K_d of 12 nM. Fifty microliters of [^{3}H]-WIN35,428 (~40 nM concentration) was added to each well of the hDAT assay plates, targeting a final [^{3}H]-WIN35428 concentration of 10 nM. Twenty microliters of binding buffer alone in the assay plate defined the total binding, whereas binding in the presence of 10 µM indatraline defined nonspecific binding. Frozen NET, SERT, or DAT membrane stocks were thawed and resuspended to a concentration of approximately 0.04 mg protein/ml binding buffer (1:1 diluted in H_2O) using a polytron tissue homogenizer. The membrane homogenates (40 µg/ml) were then lightly mixed for 5–30 min with polyvinyl toluene (PCT) wheat germ agglutinin-coated scintillation proximity assay (WGA-SPA; Amersham Biosciences) beads at 7.7 mg beads/ml homogenate. One hundred thirty microliters of the membrane/bead mixture were added to each well of the assay plate that contained radioligand and test compounds (final volume in each well, 200 µl) to start the assay, which was incubated for approximately 2 h at room temperature with agitation. The assay plates were then counted in the PVT SPA counting mode of a Packard Topcount. Fifty microliters of the [^{3}H]-nisoxetine, [^{3}H]-citalopram, or [^{3}H]-WIN35428 stocks were counted in 5 ml of ReadySafe scintillation cocktail (Beckman Industries) on a Packard 1900CA liquid scintillation counter to determine the total counts added to the respective assays. Non-linear regression was used to fit the data to sigmoid curves and determine IC_{50} values for binding and uptake. K_i values for binding and uptake were calculated using the following Cheng-Prusoff equation: $K_i = IC_{50}/(1+ [S]/K_m)$.[29].

Monoamine uptake in vitro. Two different methodological approaches were used to assess the effects of the drug on monoamine uptake. Method A used centrifugation through silicon oil, and method B used buffer to stop the reaction and wash the cells. *Method A:* The SERT, NET, and DAT functions were evaluated in human HEK 293 cells that stably expressed human SERT, NET, and DAT. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Zug, Switzerland) with 10% fetal bovine serum and 250 µg/ml geneticine. The cells (100 µl, 4×10^6 cells/ml) were incubated for 10 min with 25 µl uptake buffer (9.99 mM L-glucose, 0.492 mM $MgCl_2$, 4.56 mM KCl, 119.7 mM NaCl, 0.7 mM NaH_2PO_4, 1.295 mM NaH_2PO_4, 0.015 mM sodium bicarbonate, and 1 mg/ml ascorbic acid for [^{3}H]-DA uptake) that contained various concentrations of inhibitor at 25°C. Fifty microliters of 5 nM (final concentration) [^{3}H]-5-HT (80 C_i/mmol; Anawa), [^{3}H]-NE (14.8 C_i/mmol; Perkin-Elmer), or [^{3}H]-DA (13.8 C_i/mmol; Perkin-Elmer) was added to start uptake. Uptake was stopped after 10 min, and radioactivity was measured as described below for 5-HT and NE release. Cell integrity after MDMA treatment was confirmed by the Toxilight toxicity assay (Lonza, Basel, Switzerland). The data were fit by non-linear regression, and K_m, EC_{50}, and E_{max} values were calculated using Prism (GraphPad, San Diego, CA). Preliminary experiments showed that the accumulation of 5-HT and NE by the cells was time-dependent and complete after 5 min for both 5-HT and NE, respectively. The 5-HT and NE transport velocity was concentration-dependent and could be described by Michaelis-Menten kinetics. The K_m values were 489±147 nM, 450±125 nM, and 1707±297 nM for 5-HT, NE, and DA, respectively. 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. Nonspecific uptake was <10% of total uptake. *Method B:* Ligand potencies to inhibit [^{3}H]-DA, [^{3}H]-5-HT, and [^{3}H]-NE uptake via the human DAT, SERT and NET recombinantly expressed in HEK 293 cells were determined. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Zug, Switzerland) with 10% fetal bovine serum and 250 µg/ml geneticine in cell culture flasks. One day before the experiment, the cells were seeded in a volume of 110 µl at a density of 0.3 million cells/ml in 96-well plates (Packard) and incubated at 37°C and 5% CO_2 overnight. On the day of the uptake experiment, the 96-well plates that contained the cells were washed with Krebs Ringer bicarbonate buffer (Sigma-Aldrich, Buchs, Switzerland). Test compounds (100 µl, diluted in Krebs Ringer bicarbonate buffer) were added to the microtiter plates and incubated at 37°C for 30 min. Afterward, 50 µl [^{3}H]-DA (35–54 C_i/mmol; Perkin-Elmer; final concentration, 100 nM), [^{3}H]-5-HT (28–100 C_i/mmol; Perkin-Elmer; final concentration, 10 nM), or [^{3}H]-NE (5.3–14 C_i/mmol; Perkin-Elmer; final concentration, 100 nM) were added to DAT-, SERT-, and NET-containing cells, respectively, and incubated for 10 min at 37°C. Extracellular [^{3}H]-DA, [^{3}H]-5-HT, and [^{3}H]-NE were removed, and the plates were washed twice with Krebs Ringer bicarbonate buffer. Nonspecific uptake was determined in the presence of 10 µM indatraline. Scintillant (Microscint 40, 250 µl) was dispensed to every well, and radioactivity was determined at least 1 h later on the Packard Topcount plate reader. The data were fit by non-linear regression, and the IC_{50} was calculated using Excel (Microsoft, Redmont, CA, USA). The compounds were tested at least three times. The K_m values were 1082 nM for [^{3}H]-5-HT and >10000 nM for [^{3}H]-DA and [^{3}H]-NE.

5-HT and NE release in vitro. Transporter-mediated MDMA- and MDA-induced 5-HT and NE release was evaluated using [^{3}H]-5-HT- and [^{3}H]-NE-preloaded HEK 293 cells that stably expressed human SERT and NET, respectively. The procedures were adapted from previous studies [2,3]. SERT- or NET-expressing cells (100 µl, 4×10^6 cells/ml) were incubated at 25°C for 10 min with 50 µl of 5 nM (final concentration) [^{3}H]-5-HT or 10 nM [^{3}H]-NE solutions, respectively. Steady-state load with radiolabeled substrate was reached within 5 min and remained stable for 60 min for both cell lines. Duloxetine or other transporter inhibitors (5 µl) were added after 10 min, and the release of [^{3}H]-5-HT and [^{3}H]-NE was then initiated after another 2 min by the addition of MDMA, MDA, or buffer (25 µl). The release reaction was stopped after 10 and 30 min for [^{3}H]-5-HT and [^{3}H]-NE, respectively. The release times were based on the evaluation of the release-over-time curves for MDMA and MDA. The release of [^{3}H]-5-HT and [^{3}H]-NE was complete within 5 and 25 min, respectively, when a new steady state was reached and maintained for 30 min. To stop the release reaction and wash the cells, 100 µl of the cell suspension was transferred to 0.5 ml microcentrifuge tubes that contained 50 µl of 3 M KOH and 200 µl silicon oil (1:1 mixture of silicon oil types Ar20 and Ar200; Wacker Chemie, Munich, Germany) and centrifuged in a tabletop microfuge (Eppendorf, Basel, Switzerland) for 3 min at 13,200 rpm. This transports the cells through the silicon oil layer to the KOH layer, thereby separating the cells from the buffer, which remains on top of the silicon oil layer [30]. The centrifuge tubes were then transferred to liquid nitrogen. The amount of

tracer that remained in the cells was quantified by cutting the frozen centrifuge tube above the KOH/oil interface and putting the tip of the tube with the cell pellet in a scintillation vial that contained 500 µl lysis buffer (0.05 M TRIS-HCl, 50 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40 substitute in water). The samples were then shaken for 1 h on a rotary shaker, and 7 ml of scintillation fluid (Ultimagold, Perkin Elmer, Schwerzenbach, Switzerland) was added. Cell-associated radioactivity was then counted. The silicon oil assay allowed for the precise termination of the transport/release process and an effective cell wash. The experimental control condition (100% retained) was defined as the [^{3}H]-5HT or [^{3}H]-NE that remained in the cells when buffer and duloxetine were added without MDMA or MDA. A second control condition (100% release) was defined as the [^{3}H]-5-HT or [^{3}H]-NE released by 100 µM tyramine [6]. Data analysis using either of the two control conditions yielded similar results, and the data are presented as release expressed as the percentage of monoamine retained. Dose-response curves were generated using 9–11 concentrations of MDMA/MDA. Nonspecific binding/uptake was determined using preincubation with 10 µM fluoxetine for SERT cells and 10 µM nisoxetine for NET cells before incubation with radioligands and was <3% of total activity. All data points were derived from at least three independent experiments, each assayed in triplicate. The data were fit by non-linear regression, and EC_{50} and E_{max} values were calculated using Prism (GraphPad, San Diego, CA).

Ex vivo Binding to Monoamine Transporters

Plasma samples for assessing *ex vivo* binding to monoamine transporters were collected 120 min after MDMA/placebo administration. We determined the potencies of the plasma to inhibit [^{3}H]-nisoxetine, [^{3}H]-citalopram, and [^{3}H]-WIN35,428 binding to NET, SERT, and DAT, respectively, according to the method described previously [10]. IC_{50} values were calculated as a percentage of the plasma sample dilutions required to obtain 50% of the maximum effect. Indatraline (10 µM) in human plasma was used to achieve 100% inhibition. Undiluted plasma samples were set at 100%. Thus, an IC_{50} of 10% indicates that a 10-fold diluted plasma sample displaced 50% of the radioligand.

Statistical Analyses

Pharmacodynamics. Clinical data values were transformed to differences from baseline. Peak effects (E_{max}) were determined for repeated measures. E_{max} values were compared using General Linear Models repeated-measures analysis of variance, with drug as within-subject factor, using Statistica 6.0 software (StatSoft, Tulsa, OK). Tukey *post hoc* comparisons were performed based on significant main effects of treatment. Additional analyses of variance were performed, with period as factor to exclude period effects. Correlation analyses were performed using Pearson's correlations. The criterion for significance was $p<0.05$. Mean arterial pressure (MAP) was calculated from diastolic blood pressure and systolic blood pressure using the following formula: $MAP = DBP + (SBP - DBP)/3$.

Pharmacokinetics. The plasma concentration data for MDMA, MDA, HMMA, and duloxetine were analyzed using non-compartmental methods. C_{max} and t_{max} were obtained directly from the observed concentration-time curves. The terminal elimination rate constant (λ_z) was estimated by log-linear regression after semilogarithmic transformation of the data, using the last two to three data points of the terminal linear phase of the concentration-time curve of MDMA or duloxetine. Terminal elimination half-life ($t_{1/2}$) was calculated using λ_z and the equation $t_{1/2} = ln_2/\lambda_z$. The area under the plasma concentration-time curve up to 6 h ($AUC_{0\text{-}6h}$) was calculated using the linear trapezoidal rule. The $AUC_{0-\infty}$ was determined by extrapolation of AUC_{0-6h} using λ_z. The PK parameters were determined using the PK functions for Excel (Microsoft, Redmont, CA, USA). Plasma concentrations were only determined up to 6 h after MDMA administration because the aim of the study was to assess potential changes in MDMA plasma levels while relevant pharmacodynamic effects or MDMA were present. It was therefore not possible to determine $t_{1/2}$ for HMMA and MDA because of their long $t_{1/2}$, which would require sampling for an extended time.

PK-PD modeling: First, a soft-link PK-PD model was used to evaluate the *in vivo* relationship between the concentration of MDMA and subjective effect of the drug. The change in the VAS for any drug effect was used as the pharmacodynamic measure in each individual. Because we observed clockwise hysteresis in the effect-concentration relationship over time, we used PK-PD data pairs within the ascending part of the individual curves up to E_{max} or C_{max}. Our estimate of E_{max}, which should represent the maximal response portion of the dose-response curve, may already have been affected by tolerance. However, E_{max} values of 100% (scale maximum) or stable high values were reached by most subjects, indicating that tolerance was not an issue early in the effect-time curve. Based on the good brain penetration of MDMA and absence of a time lag, we assumed rapid equilibration between plasma and the central compartment (brain). A sigmoid E_{max} model was then fitted to the pooled data of all individuals: $E = E_{max} \times C_p^{h/}(EC_{50}^{h}+C_p^{h})$, in which E is the observed effect, C_p indicates the MDMA plasma concentration, EC_{50} indicates the plasma concentration at which 50% of the maximal effect is reached, E_{max} is the maximal effect, and h is the Hill slope. The sigmoid E_{max} model provided a better fit than a simple E_{max} or linear model. Data pooling was used because only few data pairs were available per subject. Non-linear regression was used to obtain parameter estimates. Second, we also used a hard-link PK-PD model to predict *in vivo* PD effects based on the *in vitro* concentration-response data linked to the observed individual *in vivo* PK. The *in vitro* concentration-response relationship was described by a sigmoidal dose-response variable slope model fitted to the effects of MDMA on 5-HT or NE release using non-linear regression (Prism, GraphPad, San Diego, CA). The equation was the following: $E = E_{max}/(1+10^{(LogEC50-C)\times h})$, in which C denotes the concentration of MDMA in the assay, and h denotes the Hill slope. The *in vitro* effect-concentration relationship was determined for MDMA-induced 5-HT and NE release separately, and separate PD predictions were derived for each model. Similar to the soft-link PK-PD model, a single compartment PK model (plasma = brain concentration) was used, and only ascending PK or PD values were included. The *in vivo* data were linked to the PK of each individual, and a mean predicted effect-time curve was established.

Results

Pharmacodynamics (PD)

Duloxetine markedly reduced the psychotropic and cardiostimulant responses to MDMA in humans. Duloxetine decreased all aspects of MDMA's subjective effects in the VASs [8,10], including psychostimulant effects such as feelings of "good drug effects," "drug liking," "drug high," and "stimulation" (Table 1; Fig. 2b-d) but also so-called "entactogenic" or "empathogenic" MDMA-typical effects [31,32] such as feelings of being "open," "closer to others," and more "talkative" (Table 1; Fig. 2e and f). In the AMRS [21], duloxetine prevented MDMA-induced increases in "well-being," "emotional excitation," and "extroversion"

Table 1. Pharmacodynamic peak drug effects.

		Placebo-placebo	Duloxetine-placebo	Placebo-MDMA	Duloxetine-MDMA	$F_{3,45}$ =	$p<$
Visual Analog Scales							
Any drug effect	E_{max}	3.81±3.62	6.00±2.52$^{\#\#\#}$	86.69±3.57***	33.19±7.74*** $^{\#\#\#}$	74.47	0.001
Good drug effect	E_{max}	4.56±4.37	8.75±5.01$^{\#\#\#}$	89.38±4.67***	40.56±9.50*** $^{\#\#\#}$	42.89	0.001
Drug liking	E_{max}	4.13±4.06	7.56±4.43$^{\#\#\#}$	90.69±4.82***	38.38±8.91*** $^{\#\#\#}$	52.60	0.001
Drug high	E_{max}	1.94±1.94	4.81±2.93$^{\#\#\#}$	87.81±4.85***	28.94±9.35** $^{\#\#\#}$	55.45	0.001
Stimulated	E_{max}	4.13±1.94	5.13±2.45$^{\#\#\#}$	76.31±6.84***	22.25±7.65$^{\#\#\#}$	46.25	0.001
Open	E_{max}	1.38±0.94	0.38±0.38$^{\#\#\#}$	32.16±4.29***	6.00±3.26$^{\#\#\#}$	36.88	0.001
Closeness	E_{max}	0.00±0.00	0.00±0.00$^{\#\#\#}$	27.31±3.87***	4.63±2.49$^{\#\#\#}$	37.32	0.001
Talkative	E_{max}	1.19±0.81	0.31±0.31$^{\#\#\#}$	28.81±5.12***	10.69±3.73$^{\#\#\#}$	21.13	0.001
Adjective Mood Rating Scale							
Well-being	E_{max}	1.66±0.49	0.38±0.16$^{\#\#\#}$	7.06±1.01***	3.56±1.08$^{\#\#}$	18.0	0.001
Emotional excitation	E_{max}	0.69±0.35	0.69±0.27$^{\#\#\#}$	4.94±0.97***	1.31±0.37$^{\#\#\#}$	14.7	0.001
Extroversion	E_{max}	0.63±0.24	0.38±0.16$^{\#\#\#}$	3.50±0.61***	1.44±0.43$^{\#\#\#}$	17.5	0.001
Introversion	E_{max}	0.38±1.56	1.13±0.30	2.62±0.65**	1.69±0.59	5.4	0.01
Dreaminess	E_{max}	0.63±0.33	1.35±0.35	2.94±0.66**	1.81±0.48	4.1	0.05
Activity	E_{min}	−1,88±0.50	−2.69±0.69	−4.69±1.04*	−2.81±0.78	2.6	0.06
Circulating catecholamines							
Epinephrine (nM)	E_{max}	0.42±0.12	0.46±0.10	0.50±0.12	0.26±0.10		ns
Norepinephrine (nM)	E_{max}	−0.22±0.13	−0.18±0.07$^{\#\#\#}$	0.44±0.12***	−0.19±0.10$^{\#\#\#}$	14.7	0.001
Physiologic effect							
SBP (mm Hg)	E_{max}	8.56±1.75	6.19±1.42$^{\#\#\#}$	29.94±3.41***	10.94±1.58$^{\#\#\#}$	24.6	0.001
DPB (mm Hg)	E_{max}	6.25±1.25	6.00±0.97$^{\#\#\#}$	22.13±2.08***	9.22±1.57$^{\#\#\#}$	23.3	0.001
MAP (mm Hg)	E_{max}	5.80±1.27	5.11±1.01$^{\#\#\#}$	21.76±2.73***	8.54±1.46$^{\#\#\#}$	20.3	0.001
Heart rate (beats/min)	E_{max}	9.19±1.29	5.06±1.27$^{\#\#\#}$	26.06±2.77***	11.09±1.55$^{\#\#\#}$	25.5	0.001
Body temperature (°C)	E_{max}	0.23±0.04	0.19±0.04$^{\#\#\#}$	0.54±0.07**	0.39±0.08	7.3	0.001
List of Complaints (total score)							
Acute adverse effects	at 3 h	−0.06±0.52	−1.81±1.09$^{\#\#\#}$	5.56±1.72**	−1.25±1.49$^{\#\#}$	29.5	0.001
Sub-acute adverse effects	at 24 h	−1.00±0.58	−2.88±1.35$^{\#\#}$	3.88±1.09*	−0.38±1.32$^{\#}$	24.6	0.001
Ex vivo binding ($IC_{50\%}$)							
NET		>25	14.3±0.6*** $^{\#\#}$	23.4±0.7	13.7±0.7*** $^{\#\#\#}$	20.4	0.001
SERT		>25	1.5±0.2 *** $^{\#\#\#}$	>25	1.4±0.2 *** $^{\#\#\#}$	243.1	0.001
DAT		>25	>25	>25	>25		

Values are mean±SEM of changes from baseline of 16 subjects. *p<.05, **p<.01, and ***p<.001 vs. Placebo-placebo. $^{\#}$p<.05, $^{\#\#}$p<.01, $^{\#\#\#}$p<.001 vs. Placebo-MDMA. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. IC50%, inhibition constant calculated as % of plasma sample dilution with undiluted plasma set as 100%; NET, norepinephrine transporter; SERT, SERT, serotonin transporter; DAT, dopamine transporter; ns, nonsignificant.

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(Fig. 3). In the 5D-ASC [22,23], duloxetine robustly reduced MDMA's effects on the total ASC score ($p<0.001$) and in all three main dimensions of the scale (main effect of drug: $F_{3,45} = 26.2$, 32.6, 5.67, and 26.6 for ASC, OB, AED, and VR, respectively; all $p<0.001$; Fig. 4). Duloxetine prevented the MDMA-induced increase in circulating plasma NE levels, an endocrine marker for sympathetic system activation (Table 1), and reduced the blood pressure and heart rate response to MDMA (Table 1; Fig. 5). MDMA-induced increases in plasma NE at 60 min correlated with elevations in MAP ($r = 0.57$, $p<0.05$) and increases in VAS scores for "good drug effects," "liking," "open" ($r = 0.65$, 0.69, 0.77 and 0.63, respectively; all $p<0.01$), supporting the modulatory role of NE in these effects of MDMA. ANOVAs with period as factor showed no effect of treatment order, confirming the absence of period effects.

Pharmacokinetics

The robust decrease in the PD response to MDMA after duloxetine was not the result of a pharmacokinetic interaction between duloxetine and MDMA because duloxetine increased exposure to MDMA. MDMA and duloxetine are both substrates and inhibitors of CYP 2D6 [16]. The moderate CYP 2D6 inhibitor duloxetine increased both the C_{max} and $AUC_{0\text{-}6h}$ of the CYP 2D6 substrate MDMA by 16±4% (mean ± SEM; $F_{1,15} = 12.64$, $p<0.01$) and 18±5% ($F_{1,15} = 8.95$, $p<0.01$), respectively (Fig. 6 and Table 2). Duloxetine had no effect on exposure to MDA, the active metabolite of MDMA. Duloxetine decreased the C_{max} and $AUC_{0\text{-}6h}$ of the inactive CYP 2D6-formed MDMA metabolite HMMA by 46±6% ($F_{1,15} = 70.03$, $p<0.001$) and 48±6% ($F_{1,15} = 166.10$, $p<.001$), respectively. Plasma duloxetine concentrations nonsignificantly increased beginning 1 h after

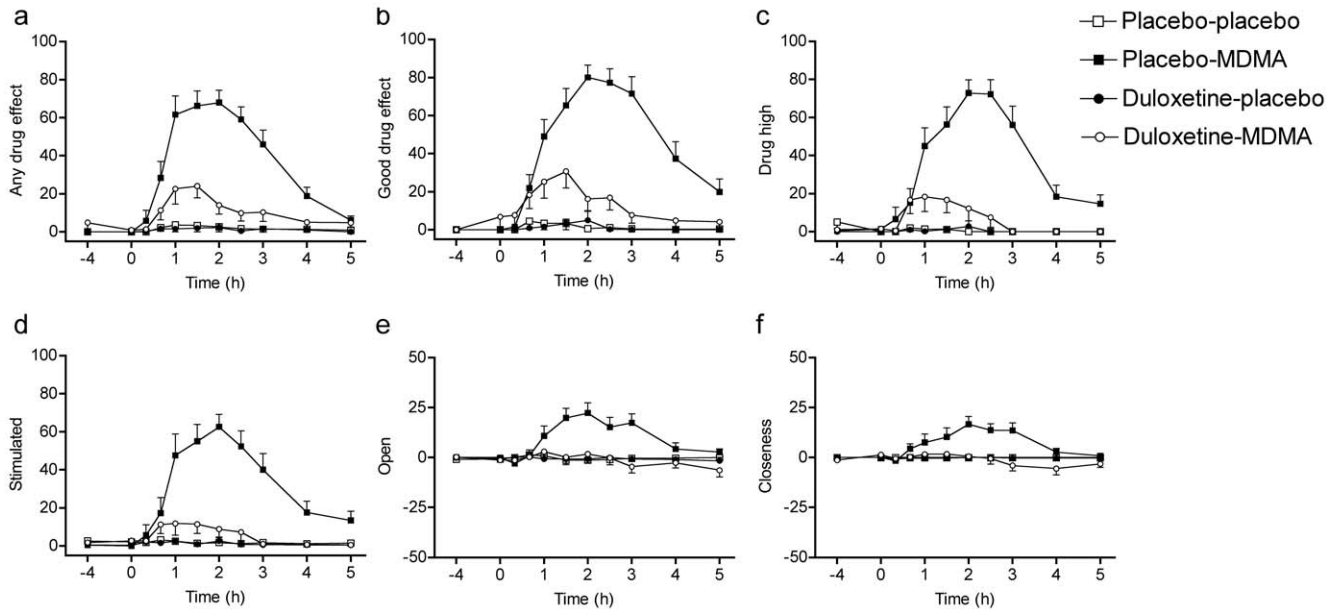

Figure 2. Duloxetine inhibited the psychotropic effects of MDMA. MDMA produced stimulant-like (**b–d**) and ''entactogenic'' (**e, f**) effects compared with placebo ($p<0.001$ for all scales). Duloxetine significantly inhibited MDMA-induced elevations in all of these subjective effects (**a–f**) ($p<0.001$ for all scales). Values are expressed as mean+SEM ($n = 16$).
doi:10.1371/journal.pone.0036476.g002

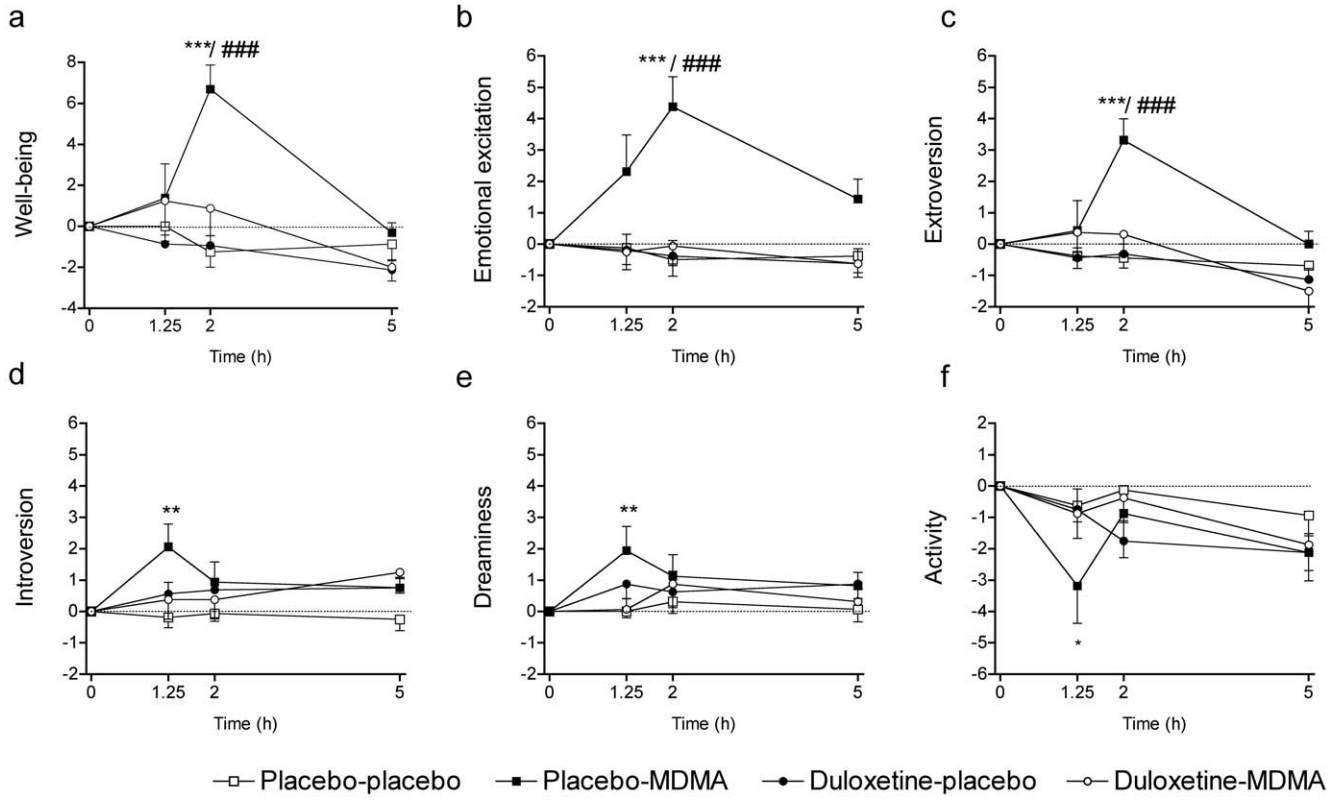

Figure 3. Duloxetine prevented the acute emotional effects of MDMA in the Adjective Mood Rating Scale. MDMA produced a state of well-being (a), emotional excitation (b), increased introversion at drug onset at 1.25 h (d), increased extroversion at 2 h (c), increased dreaminess (e), and decreased performance-oriented activity (f) (*$p<0.05$, **$p<0.01$, ***$p<0.001$, *vs*. placebo-placebo). Duloxetine prevented MDMA-induced elevations in well-being, emotional excitation, and extroversion (a-c) ($^{###}p<0.001$, placebo-MDMA *vs*. duloxetine-MDMA). Values are expressed as mean+SEM ($n = 16$).
doi:10.1371/journal.pone.0036476.g003

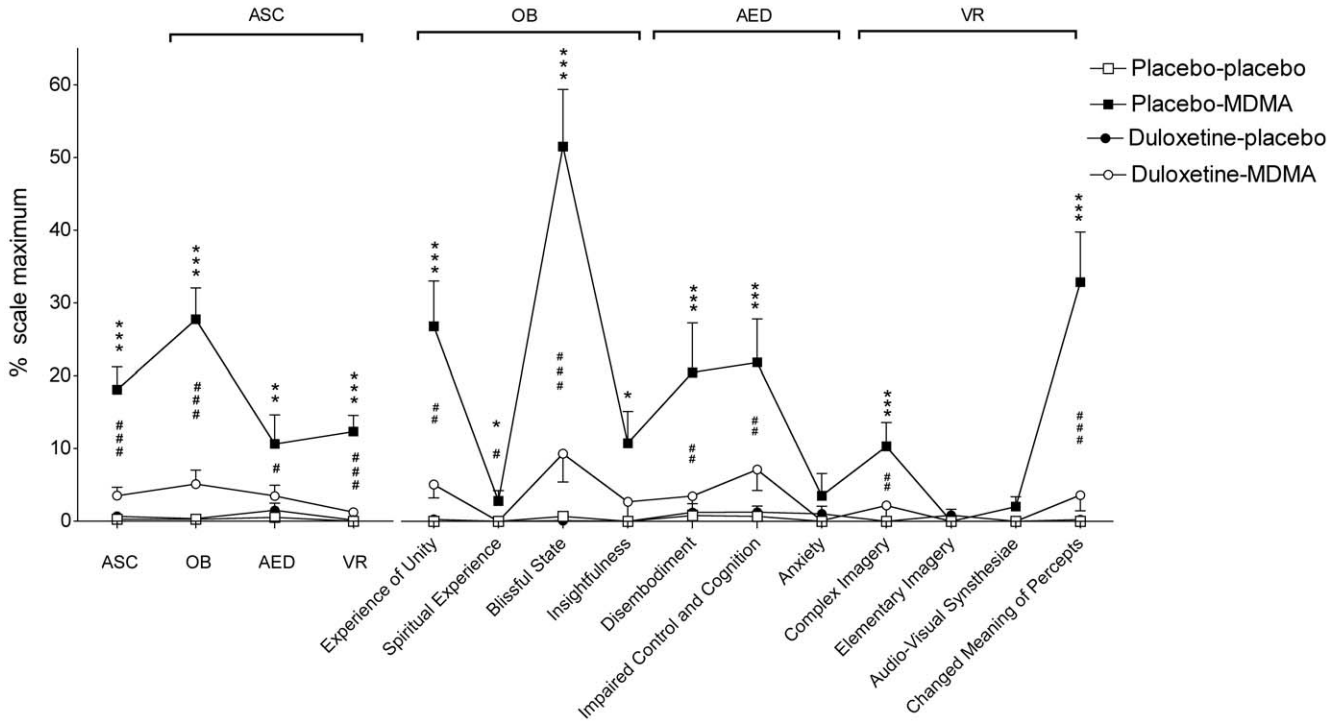

Figure 4. Duloxetine prevented the acute effects of MDMA in the Altered States of Consciousness (ASC) scale. MDMA significantly increased the ASC sum score, Oceanic Boundlessness (OB), Anxious Ego Dissolution (AED), and Visionary Restructuralization (VR) dimensions, and most of the subscales (*$p<0.05$, **$p<0.01$, ***$p<0.001$, placebo-placebo vs. placebo-MDMA). Duloxetine significantly reduced the effect of MDMA in all dimensions and subscales ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, placebo-MDMA vs. duloxetine-MDMA). Values are expressed as mean+SEM (n = 16).
doi:10.1371/journal.pone.0036476.g004

MDMA administration (Fig. 5), consistent with the inhibitory effect of MDMA on duloxetine metabolism via CYP 2D6. Interindividual differences in CYP 2D6 activity also affected the PK of MDMA. Lower CYP 2D6 function (i.e., a lower dextromethorphan:dextrorphan urine concentration ratio) was associated with a longer $t_{1/2}$ of MDMA ($r = 0.65$, $p<0.01$).

PK-PD Relationship

Fig. 7 shows the mean PD effects of MDMA plotted against simultaneous plasma concentrations at the different time points (hysteresis loops). The increases in "any drug effect" (Fig. 7a) and MAP (Fig. 7b) returned to baseline within 6 h when MDMA concentrations were still high. This clockwise hysteresis indicates that a smaller MDMA effect was seen at a given plasma concentration later in time, indicating rapid acute pharmacodynamic tolerance, which was similarly described for cocaine [33]. Duloxetine robustly reduced the physical and subjective response to MDMA, but it increased exposure to MDMA, illustrated by the downward and rightward shift of the MDMA hysteresis loops (Fig. 7).

Adverse Effects

MDMA produced adverse effects, such as sweating, difficulty concentrating, thirst, and lack of appetite, resulting in an increase in total LC scores at both 3 and 24 h after drug administration (Table 1). Duloxetine produced daytime somnolence and moderate insomnia. No severe adverse events were observed.

In vitro Studies

MDMA-induced 5-HT and NE release studies *in vitro*. MDMA was nonsignificantly more potent in releasing NE via NET than 5-HT via SERT ($IC_{50} = 0.55$ and 1.69 μM, respectively; Fig. 8; Table 3), consistent with earlier work that used human [3,34] and rat [2] transporters. MDA similarly released monoamines with EC_{50} values of 0.85 and 2.77 μM for NE and 5-HT, respectively (Fig. 8; Table 3). Thus, both amphetamines were active transporter-mediated monoamine releasers and exhibited slightly higher potency at NET than SERT. Duloxetine potently inhibited the ability of MDMA and MDA to induce 5-HT release from SERT and NE release from NET cells (Fig. 8). Duloxetine (0.1 μM) decreased the E_{max} by approximately 50% and shifted the concentration-effect curves to the right, consistent with a mixed competitive and noncompetitive mode of inhibition. A high concentration of duloxetine (10 μM) completely blocked the effects of MDMA and MDA (Fig. 8). We then compared the inhibitory effect of duloxetine on MDMA-induced monoamine release to the inhibitory effects of the selective SERT inhibitor citalopram and selective NET inhibitor reboxetine, each of which have been shown to attenuate some of the effects of MDMA in humans [7,10]. The potencies of duloxetine and citalopram to inhibit MDA- and MDMA-induced 5-HT release were similar (Fig. S1; Table 3). The potencies of duloxetine and reboxetine to block MDMA-induced NE release were also similar (Fig. S1; Table 3). These *in vitro* data indicate that duloxetine inhibited both SERT and NET similarly to citalopram and reboxetine, respectively.

PK-PD and *in vitro-in vivo* relationship. Duloxetine mainly affected the E_{max} of MDMA in the *in vivo* PK-PD relationship of MDMA (Fig. 9a) consistent with a primarily

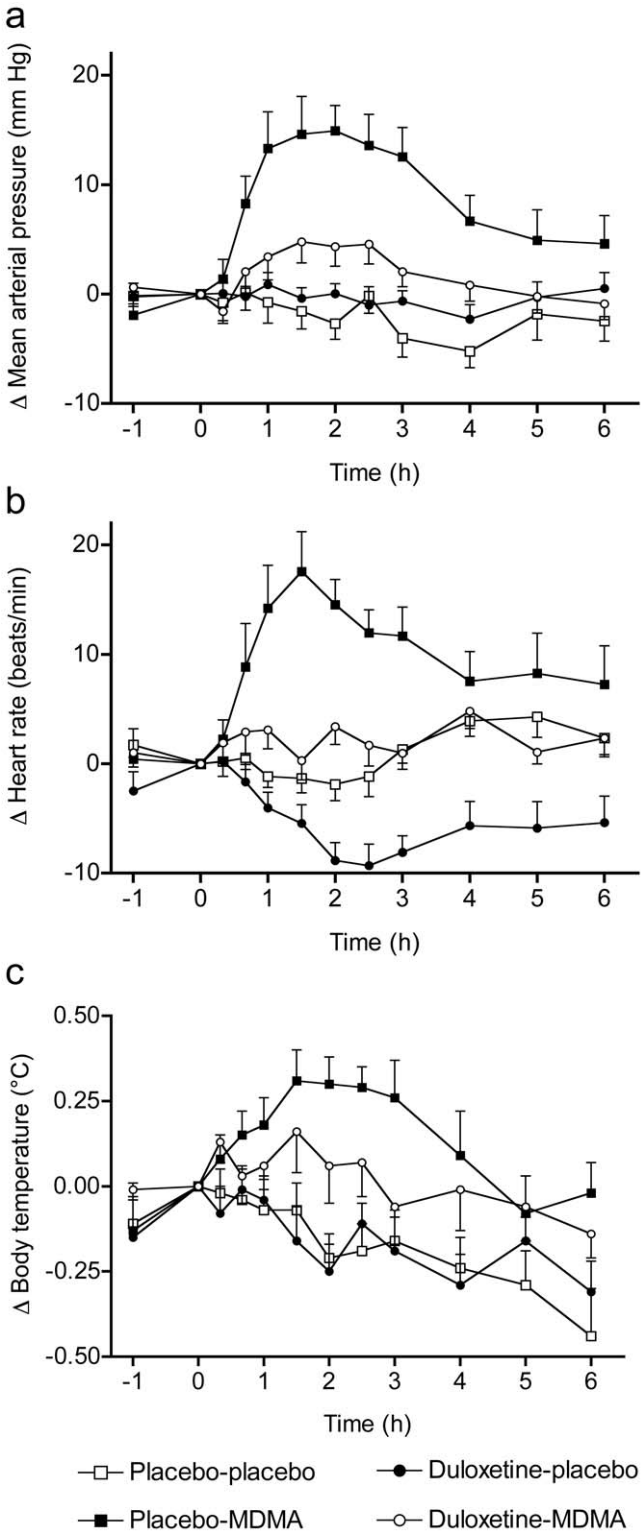

Figure 5. Duloxetine reduced the cardiostimulant response to MDMA. Duloxetine reduced the elevations in mean arterial blood pressure (**a**) and heart rate (**b**) in response to MDMA. Duloxetine also nonsignificantly lowered the MDMA-induced increase in body temperature (**c**). Values are expressed as mean+SEM of 16 subjects.
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noncompetitive mode of inhibition and similar to the effect of duloxetine on monoamine release produced by MDMA *in vitro*. Duloxetine decreased the E_{max} from 93.8±7.3% to 20.8±4% for placebo-MDMA compared with duloxetine-MDMA, respectively. The EC_{50} values were 92.5±7.6 ng/mL (0.48 μM) and 83.8±25 ng/mL (0.43 μM) for placebo-MDMA and duloxetine-MDMA, respectively. The EC_{50} of the PK-PD curve of placebo-MDMA in humans was 74 ng/ml (0.38 μM), similar to the EC_{50} values of MDMA to release 5-HT and NE *in vitro*. The plasma concentrations of duloxetine (C_{max} = 112 ng/ml or 0.38 μM) were also in the range of the concentrations that reduced MDMA-induced 5-HT and NE release *in vitro*. To relate our *in vitro* data to the PD of MDMA in humans, we linked the concentration-effect relationship of the *in vitro* effect of MDMA on 5-HT and NE release to the individual concentration-time curves of our subjects (Fig. 9b). The observed effect-time curve for MDMA in humans was predicted well by the *in vitro* NE release model, assuming similar concentrations in plasma and brain and no time lag. The 5-HT release model fitted, but 2- to 10-fold higher MDMA concentrations in the brain than in plasma would be needed to obtain similar pharmacodynamic effects as NE. The higher potency of MDMA to release NE *vs.* 5-HT *in vitro* also predicted that NE release occurred at lower MDMA plasma and brain concentrations and therefore sooner after MDMA administration, playing a predominant role during the initial drug effect (i.e., rush, stimulant effect). 5-HT release becomes relatively more important later in time and predominantly mediates "entactogenic" effects, including feelings of being open and closer to others, that prevail later. The model predicted that the half-maximal effects would be reached at 40±2 min and 70±14 min for NE and 5-HT release, respectively (Fig. 9b). The observed half-maximal subjective drug effect of MDMA was reached 44±4 min after drug administration. At that time, the models predicted 4 (3–6)-fold higher NE release compared with 5-HT release, consistent with the view of a primary role for NE in the early effects of MDMA.

Monoamine transporter binding *in vitro*. The binding of MDMA and MDA to monoamine transporters was weak (Table 4) compared with the high potency of MDMA to release 5-HT and NE. The binding profile of MDMA was consistent with other binding studies that used human transporters [3] but different from studies that used rat transporters [17]. Duloxetine showed more than 100-fold higher affinity for both SERT and NET compared with the affinity of MDMA for these transporters in the same assay, supporting our approach of using duloxetine to prevent MDMA from interacting with SERT and NET (Table 4).

Monoamine uptake inhibition *in vitro*. MDMA inhibited NET three-fold more potently than SERT, consistent with previous studies that used human transporters [3,35] but in contrast to data derived from mouse and rat transporters [17,35,36] (Table 5). MDA was equally potent to MDMA in inhibiting NET and SERT. Both MDMA and MDA showed low potency to inhibit DAT. Duloxetine was more potent in inhibiting SERT than NET (Table 5), which was expected [13]. Because the selective SERT inhibitor citalopram and selective NET inhibitor reboxetine have previously been shown to attenuate the psychological effects of MDMA [7,10], we compared duloxetine with these inhibitors. Duloxetine exhibited similar potency as citalopram to inhibit SERT but 2- to 5-fold lower potency as reboxetine to inhibit NET (Table 5).

Ex vivo Binding Studies

The ability of duloxetine to block monoamine transporters in our study was confirmed with an *ex vivo* assay, in which plasma from duloxetine-treated subjects inhibited *ex vivo* radioligand binding to SERT and NET but not DAT (Table 1). We also found a 10-fold higher affinity for SERT compared with NET,

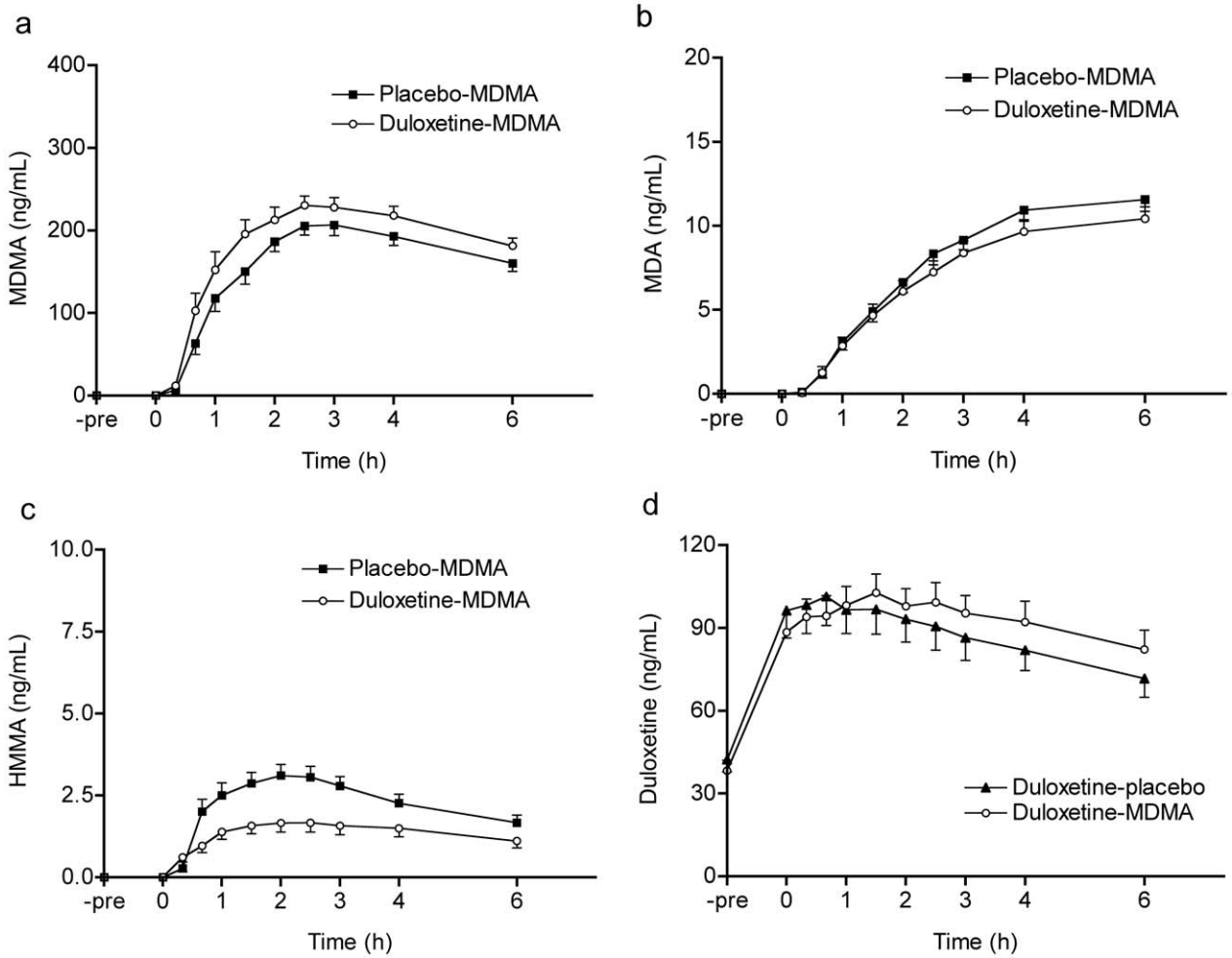

Figure 6. Duloxetine increased MDMA exposure. Pharmacokinetics of MDMA, MDA, HMMA, and duloxetine (**a–d**). Duloxetine was administered 16 h and 4 h before MDMA, which was administered at the 0 h time point. Duloxetine increased the C_{max} and AUC_{0-6} of MDMA (**a**), had no significant effect on MDA exposure (**b**), and decreased the C_{max} and AUC_{0-6} of HMMA (**c**). Plasma duloxetine concentrations were similar in the duloxetine-placebo and duloxetine-MDMA groups before MDMA administration (at –4 h and 0 h). Duloxetine concentrations increased 1 h after MDMA administration in the duloxetine-MDMA *vs*. duloxetine-placebo group (**d**). Values are expressed as mean±SEM of 16 subjects. MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine.
doi:10.1371/journal.pone.0036476.g006

Table 2. Pharmacokinetic parameters of MDMA, MDA, HMMA, and duloxetine.

	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-6} (ng/ml h)	$AUC_{(0-\infty)}$ (ng/ml h)
MDMA					
Placebo-MDMA	221.31±11.63	2.34±0.19	8.17±0.74	952.75±45.89	2908.55±275.64
Duloxetine-MDMA	253.63±13.60**	2.66±0.29	7.14±0.40	1106.87±57.22**	2915.28±154.27
MDA					
Placebo-MDMA	11.75±0.70	5.50±0.22	–	46.60±3.02	–
Duloxetine-MDMA	10.67±0.72	5.25±0.30	–	41.95±3.38	–
HMMA					
Placebo-MDMA	3.36±0.34	1.84±0.17	–	13.57±1.58	–
Duloxetine-MDMA	2.00±0.38***	1.89±0.25	–	8.14±1.45***	–
Duloxetine					
Duloxetine-placebo	106.77±10.25	5.14±0.29	10.97±1.04	799.88±74.40	1960.18±229.54
Duloxetine-MDMA	111.69±7.06	5.95±0.39	11.37±1.43	814.31±52.73	2189.45±297.99

C_{max}, maximum plasma concentration; T_{max}, time from drug administration to maximum plasma concentration; $AUC_{0-\infty}$, area under concentration-time curve extrapolated to infinity. HMMA, 4-hydroxy-3-methoxymethamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine.
**p<.01, ***p<.001, vs. Placebo-MDMA. Values are mean±SEM (n =16).
doi:10.1371/journal.pone.0036476.t002

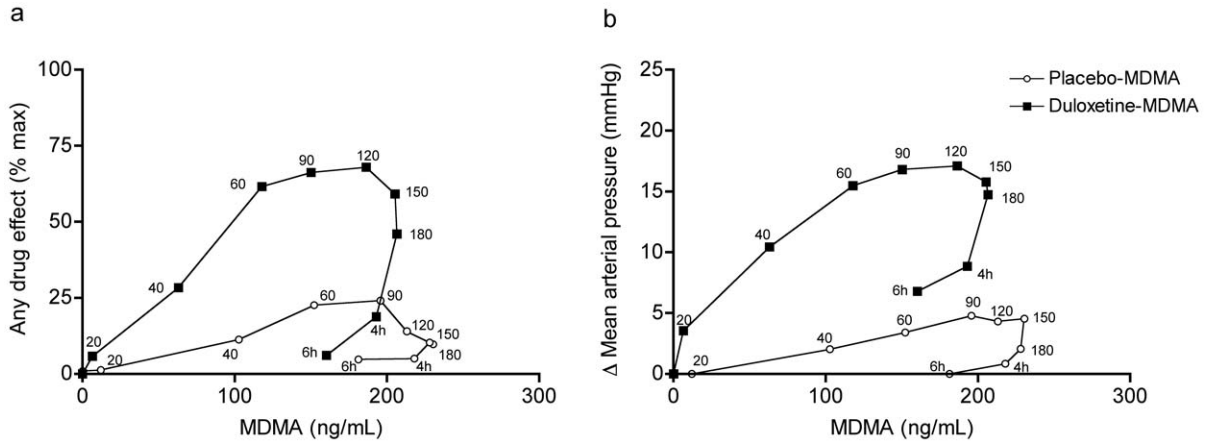

Figure 7. Pharmacokinetic-pharmacodynamic (PK-PD) relationship. MDMA effects are plotted against simultaneous MDMA plasma concentrations (**a, b**). The time of sampling is noted next to each point in minutes or hours after MDMA administration. The clockwise hysteresis indicates acute tolerance to the effects of MDMA. Duloxetine pretreatment markedly reduced physical and subjective responses to MDMA in the hysteresis loops (**a, b**).
doi:10.1371/journal.pone.0036476.g007

which was previously shown [13] and consistent with the *in vitro* profile of duloxetine. We calculated the duloxetine concentration in the plasma samples using the K_i values of duloxetine for SERT and NET binding (Table 2) and the IC_{50} values derived from the *ex vivo* binding in the duloxetine-placebo group (Table 1). The values (mean ± SE) obtained were 388±36 nM and 576±44 nM duloxetine using SERT and NET binding, respectively, which was well in agreement with the duloxetine plasma concentrations determined by LC-MS/MS (314±2.5 nM). Plasma from MDMA-treated subjects did not differ from placebo-treated subjects with regard to *ex vivo* radioligand binding to monoamine transporters (Table 1). This finding is consistent with the relatively low *in vitro* binding affinity of MDMA, which does not reflect the high pharmacological activity of the drug. Our assay assessed binding to

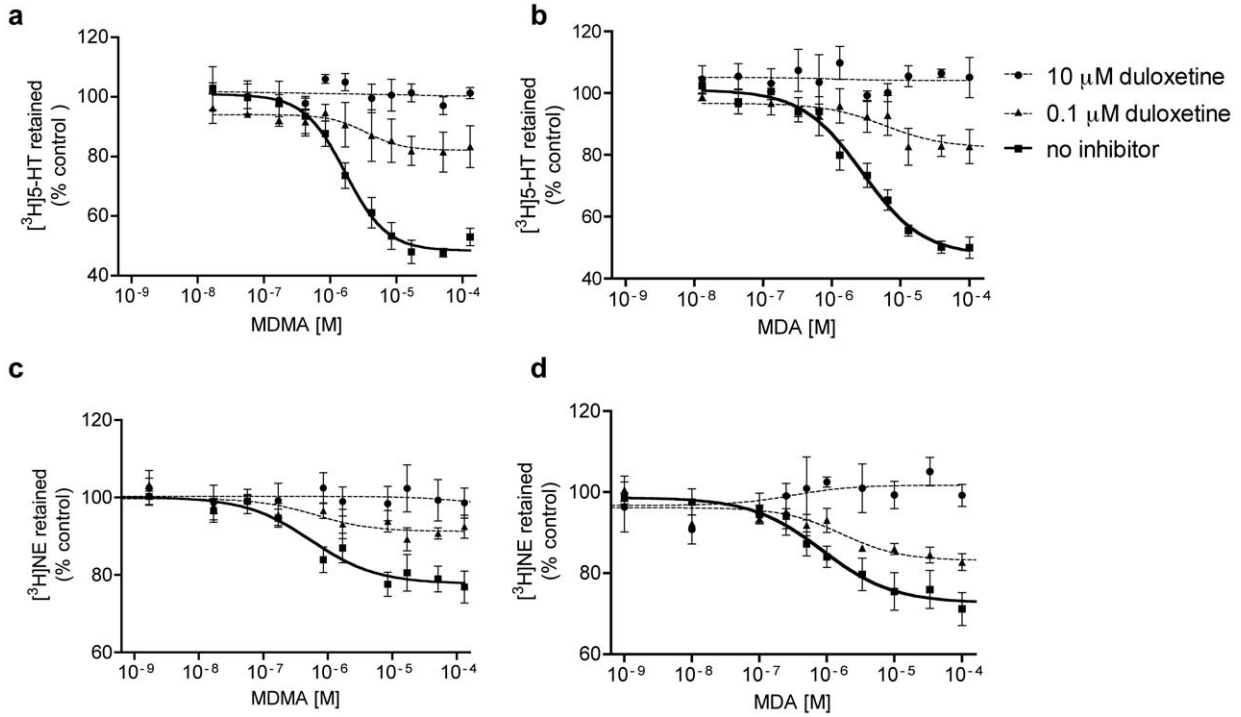

Figure 8. Duloxetine blocked MDMA- and MDA-induced 5-HT and NE efflux. Duloxetine inhibited SERT-mediated 5-HT release by MDMA (**a**) and MDA (**b**). Duloxetine also inhibited NET-mediated NE release by MDMA (**c**) and MDA (**d**). Values are expressed as mean ± SEM (n = 3–6) of retained radiolabeled substrate following incubation with various concentrations of MDMA and MDA.
doi:10.1371/journal.pone.0036476.g008

Table 3. Inhibition of MDMA-induced 5-HT or NE release by different inhibitors.

	SERT		NET	
	EC_{50} (μM) (95% CI)	E_{max}, % retained, (95% CI)	EC_{50} (μM) (95% CI)	E_{max}, % retained, (95% CI)
MDMA alone	1.69 (1.07–2.66)	48 (42–55)	0.55 (0.17–1.81)	78 (73–82)
MDMA plus 0.1 μM duloxetine	3.51 (0.46–27)	82 (75–90)	0.59 (0.02–19)	90 (84–97)
MDMA plus 0.1 μM citalopram	3.17 (1.89–5.31)	72 (68–77)	na	na
MDMA plus 0.1 μM reboxetine	na	Na	3.35 (0.63–179)	78 (56–102)
MDA alone	2.77 (1.78–4.30)	48 (41–54)	0.85 (0.29–2.55)	73 (67–79)
MDA plus 0.1 μM duloxetine	6.86 (0.5–100)	83 (77–89)	2.06 (0.35–12.12)	80 (73–87)
MDA plus 0.1 μM citalopram	5.0 (1.28–19.6)	59 (44–75)	na	na

95% CI, 95% confidence interval; na, not assessed.
doi:10.1371/journal.pone.0036476.t003

the SERT and NET binding site for [^{3}H]-citalopram and [^{3}H]-nisoxetine, respectively. A possible explanation for the low affinity of MDMA in this assay could be a binding site for MDMA that is different from citalopram and nisoxetine at SERT and NET, respectively, consistent with the noncompetitive mode of inhibition of the MDMA-induced 5-HT and NE release by duloxetine.

Discussion

The present study showed that the dual SERT and NET inhibitor duloxetine markedly decreased the psychotropic and cardiovascular responses to MDMA in human subjects, confirming and extending previous work with selective SERT [7,8,9] and NET [10] inhibitors. The inhibition of the effect of MDMA by duloxetine in humans was pronounced and primarily noncompetitive. *In vitro*, duloxetine similarly blocked the interactive effects of MDMA with SERT and NET to release 5-HT and NE. The present findings provide further support for a central role of SERT and NET as targets of MDMA with regard to its acute effects in humans. Previous clinical data indicated that 5-HT release primarily mediates the MDMA-typical "empathogenic" mood effects of MDMA [7], whereas NE release may be responsible for the stimulant and cardiovascular effects of the drug [10]. In the present study, dual inhibition of 5-HT and NE release robustly blocked both aspects of the MDMA effect, consistent with the role of both 5-HT and NE. The precise mode of interaction of amphetamine derivatives, including MDMA, with monoamine transporters remains to be elucidated and may involve the exchange of amphetamine with the transmitter, channel-like conformational changes of the transporter [37], or transporter internalization [38,39,40], MDMA is structurally similar to 5-HT, and a common binding site has been proposed in transmembrane domain 6 of SERT [41]. A distinct binding site was found for SERT inhibitors, including citalopram and fluoxetine, proximal to the 5-HT binding site [42]. Some SERT inhibitors may therefore allosterically inhibit the interaction between MDMA and SERT to release 5-HT. Consistent with these molecular data, our study showed that duloxetine inhibited MDMA-induced 5-HT release, NE release, and the response to MDMA in humans possibly according to a noncompetitive inhibition mode. Both our *in vitro* and *in vivo* findings may indicate acute allosteric inhibition of the effects of MDMA by duloxetine. Prior work with rat brain synaptosomes showed that indatraline competitively inhibited MDMA-induced 5-HT release [5]. However, later studies indicated that many SERT inhibitors also decreased the E_{max} for different monoamine releasers, suggesting unique transporter

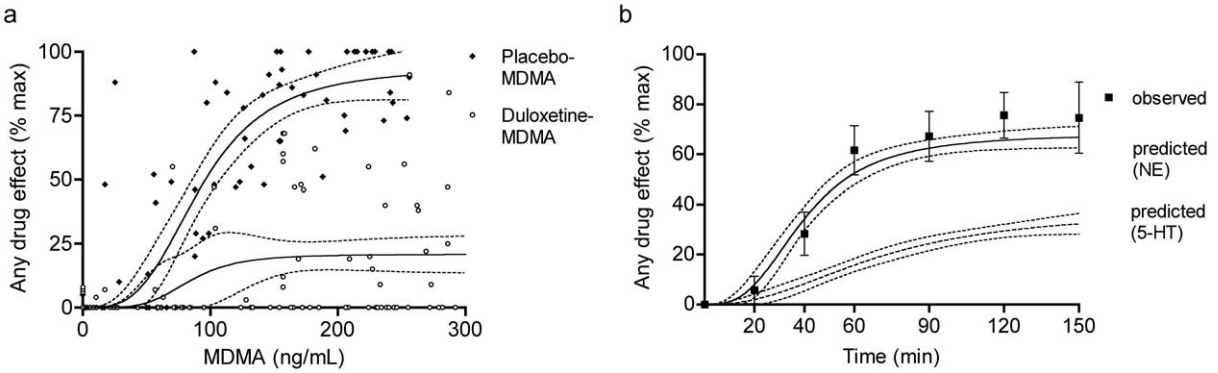

Figure 9. Pharmacokinetic-pharmacodynamic modeling. Duloxetine lowered E_{max} in the MDMA concentration-effect curve (**a**) with little effect on EC_{50}, similar to the effect of MDMA on monoamine release *in vitro*. Diamonds and circles represent concentration-effect data pairs for ascending concentrations for placebo-MDMA and duloxetine-MDMA, respectively (**a**). The solid lines show the fit of a sigmoid E_{max} PD model to the observed PK data (**a**). Dashed lines indicate the 95% confidence interval (CI) of the estimation error (**a**). NE release predicted the observed subjective effect of MDMA *in vivo* (**b**). Predicted effects are shown as curves (mean ±95% CI) that represent the fit of the *in vitro* concentration-effect data to the 16 individual plasma concentration-time curves (**b**). Observed values are expressed as mean±SEM of 16 subjects (**b**). MDMA, 3,4-methylenedioxymethamphetamine; NE, norepinephrine; 5-HT, serotonin.
doi:10.1371/journal.pone.0036476.g009

Table 4. Binding affinities to human monoamine transporters.

	SERT	NET	DAT
MDMA	13.3±0.47	22.4±14.6	6.52±2.24
MDA	18.7±2.76	17.8±4.06	26.4±4.24
Duloxetine	0.005±0.002	0.07±0.05	0.70±0.07
Reboxetine	0.24±0.02	0.015±0.01	16.2±4.91
Citalopram	0.005±0.001	5.06±3.00	21.4±10.5
Indatraline	0.02±0.008	0.03±0.02	0.01±0.01
Paroxetine	0.004±0.001	0.42±0.17	0.77±0.18

Values are mean±SD of K_i (μM) (n≥3). Radioligands were ^{3}[H]citalopram, ^{3}[H]nisoxetine, and ^{3}[H]-WIN35,428 for SERT, NET, and DAT, respectively.
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interactions for different inhibitor-releaser combinations [6]. This indicates that different SERT inhibitors may also more or less effectively reduce the effects of psychostimulants in humans. Nevertheless, several of the present findings indicate that the effect of duloxetine on the MDMA response was likely attributable to the dual inhibition of SERT and NET and not only the result of potent SERT inhibition alone. First, duloxetine blocked MDMA-induced NE release *in vitro* and MDMA-induced increases in plasma NE *in vivo*, similar to the selective NET inhibitor reboxetine [10]. Second, we documented, *ex vivo*, NET binding in plasma from duloxetine-treated subjects, and duloxetine has previously been shown to effectively inhibit NET in humans [13]. Third, potent and selective inhibition of SERT alone using citalopram in a single high dose [7], fluoxetine for 5 days [8], or paroxetine for 3 days [9] failed to block the effects of MDMA in humans to the extent seen here with dual SERT and NET inhibition. Conversely, selectively blocking NET alone also did not as effectively reduce the effects of MDMA in humans [10] as blocking both SERT and NET. The importance of NE as a modulator of the acute effects of MDMA is also supported by the fact that NE plasma levels after MDMA treatment in the present study correlated with the subjective effects and increases in blood pressure. Furthermore, we compared our *in vitro* 5-HT and NE release data to clinical data in humans and showed that the NE release link model better predicted the ascending subjective effects of MDMA in humans than the 5-HT release link model. A full assessment of the relative efficacy of SERT and NET inhibitors to prevent the effects of MDMA would require administration of SERT and NET inhibitors alone and in combination and dose-response studies. However, such studies were not ethically feasible because we did not want to expose our MDMA-naive subjects to more than two doses of MDMA in a crossover design.

The role of DA in the reinforcing effects of psychostimulants is well established, but unknown is whether DA is critical for the acute effects of MDMA. We found that MDMA exhibited higher affinity for DAT than NET or SERT *in vitro*. However, MDMA functionally exhibited significantly higher inhibition potency of the SERT and NET compared with DAT, respectively. MDMA is also more potent in releasing 5-HT and NE compared with DA *in vitro* [3], and the magnitude of 5-HT release exceeded DA release in the nucleus accumbens, striatum, and prefrontal cortex, assessed with *in vivo* microdialysis in rats [43]. DAT inhibition did not affect the acute response to MDMA in rhesus monkeys [44]. Additionally, the D_2 dopamine receptor antagonist haloperidol only weakly attenuated MDMA-induced euphoria in humans and only at doses that produced significant dysphoria [45]. Whether DAT (NET) inhibitors, such as bupropion or methylphenidate, inhibit the effects of MDMA in humans remains to be tested. Duloxetine is a potent SERT and NET inhibitor but also weak DAT inhibitor [13,46], which was confirmed in the present *in vitro* study. We cannot exclude the possibility that the relatively high dose of duloxetine used in the present study also inhibited MDMA-induced DA release. Notably, the present *ex vivo* binding studies further showed that the plasma from the subjects treated with duloxetine exhibited binding to SERT and NET but not DAT.

The transporter-independent vesicular release of monoamines could theoretically contribute to the mechanism of action of MDMA. We recently showed that this is not the case for NE because clonidine, which blocks transporter-independent vesicular NE release, did not alter the effects of MDMA in humans [12]. Additionally, MDMA did not directly stimulate the Ca^{2+}-dependent vesicular release of DA [47]. Nevertheless, MDMA may indirectly stimulate the DA system and induce the vesicular release of DA by downstream 5-HT-DA or NE-DA system interactions. For example, 5-HT release by MDMA stimulates DA release via 5-HT_2 receptor activation [48], and this indirect effect on the DA system is also prevented by SERT inhibition [49]. Thus, downstream DA system activation may be a contributing factor to MDMA-induced euphoria and the mechanism of action of psychostimulants in general, even when SERT and NET may be considered the primary pharmacological targets.

Finally, it is also possible that duloxetine induced adaptive effects on monoamine systems that reduced the response to MDMA in vivo. For example, decreases in SERT but not in NET

Table 5. Monoamine transport inhibition.

	SERT	NET	DAT
	K_i (μM) (95% CI)	K_i (μM) (95% CI)	K_i (μM) (95% CI)
MDMA*	1.40 (1.00–1.96)	0.470 (0.334–0.598)	16.7 (11.5–24)
MDA*	2.41 (1.49–3.92)	0.341 (0.253–0.461)	11 (7.5–17)
Duloxetine	0.050 (0.04–0.07)*	0.126 (0.099–0.161)*	2.26 (0.7–3.8)#
Reboxetine	2.07 (1.4–2.6)#	0.036 (0.030–0.044)*	16.4 (11.5–25.2)#
Citalopram*	0.045 (0.037–0.057)	>20	>20
Indatraline#	0.09 (0.06–0.12)	0.043 (0.03–0.06)	0.025 (0.01–0.04)
Paroxetine#	0.014 (0.01–0.02)	1.12 (0.03–1.7)	4.83 (2.4–7.3)

*method A; #method B; 95% CI, 95% confidence interval; values are significantly different ($p<0.05$) if 95% CI do not operlap.
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binding sites were documented following chronic administration of duloxetine in rats [50].

In conclusion, the present study adds to a better understanding of the mechanism of action of MDMA in humans. The data support the roles of both NE and 5-HT in the acute effects of MDMA. The robust and almost complete prevention of the effects of MDMA by duloxetine suggests that dual transporter inhibitors may be useful in the prevention of the acute and long-term consequences of MDMA and potentially other psychostimulants in addicted subjects.

Supporting Information

Figure S1 Potency and efficacy of MDMA- and MDA-induced 5-HT and NE release inhibition by duloxetine, citalopram, and reboxetine. Both duloxetine and citalopram inhibited MDMA-induced (a, c) and MDA-induced (b, d) 5-HT release *in vitro* with approximately similar potency and efficacy. The potency of duloxetine to block MDMA-induced NE release was also similar to the selective NET inhibitor reboxetine (e, f). EC_{50} and E_{max} values are shown in Table 3. Data points represent mean ± SEM.
(TIF)

Protocol S1 Trial Protocol.
(DOC)

Checklist S1 CONSORT Checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: CMH LDS MEL. Performed the experiments: CMH LDS VGN NV MD SK EG JH MCH MEL. Analyzed the data: CMH LDS MCH MEL. Wrote the paper: CMH LDS MCH MEL.

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Paper 2c

Sex Differences in the Effects of MDMA (Ecstasy) on Plasma Copeptin in Healthy Subjects

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Sex Differences in the Effects of MDMA (Ecstasy) on Plasma Copeptin in Healthy Subjects

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Background: 3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) misuse is associated with hyponatremia particularly in women. Hyponatremia is possibly due to inappropriate secretion of plasma arginine vasopressin (AVP).

Objective: To assess whether MDMA increases plasma AVP and copeptin in healthy male and female subjects and whether effects depend on MDMA-induced release of serotonin and norepinephrine. Copeptin, the C-terminal part of the AVP precursor preprovasopressin, is cosecreted with AVP and can be determined more reliably.

Methods: We used a randomized placebo-controlled crossover design. Plasma and urine osmolalities as well as AVP and copeptin levels were measured in 16 healthy subjects (eight female, eight male) at baseline and after MDMA (125 mg) administration. In addition, we tested whether effects of MDMA on AVP and copeptin secretion can be prevented by pretreatment with the serotonin and norepinephrine transporter inhibitor duloxetine (120 mg), which blocks MDMA-induced transporter-mediated release of serotonin and norepinephrine.

Results: MDMA significantly elevated plasma copeptin levels at 60 min and at 120 min compared with placebo in women but not in men. The copeptin response to MDMA in women was prevented by duloxetine. MDMA also nonsignificantly increased plasma AVP levels in women, and the effect was prevented by duloxetine. Although subjects drank more water after MDMA compared with placebo administration, MDMA tended to increase urine sodium levels and urine osmolality compared with placebo, indicating increased renal water retention.

Conclusion: MDMA increased plasma copeptin, a marker for AVP secretion, in women but not in men. This sex difference in MDMA-induced AVP secretion may explain why hyponatremia is typically reported in female ecstasy users. The copeptin response to MDMA is likely mediated via MDMA-induced release of serotonin and/or norepinephrine because it was prevented by duloxetine, which blocks the interaction of MDMA with the serotonergic and noradrenergic system. **(*J Clin Endocrinol Metab* 96: 2844–2850, 2011)**

Abuse of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) has been associated with the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) (1, 2) and symptomatic hyponatremia particularly in women (3, 4). Specifically, a case series of ecstasy-associated hyponatremia included 18 cases, of which 17 were women (4). Another larger retrospective series of ecstasy exposures reported to a poison center found hyponatremia (Na <130 mmol/liter) in 73 (38.8%) of 188 cases (3). Of the 73 cases with hyponatremia, 55 (75.3%) were women and 18 (24.7%) men (3). Thus, female sex was significantly associated with increased odds of hypona-

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Abbreviations: AVP, Arginine vasopressin; MDMA, 3,4-methylenedioxymethamphetamine; SIADH, syndrome of inappropriate secretion of antidiuretic hormone.

tremia and increased odds of associated coma among these cases (3). A small nonblinded laboratory study showed that MDMA significantly increased plasma concentrations of arginine vasopressin (AVP) at 1–4 h after controlled MDMA administration in eight healthy male volunteers (5, 6). This study provides evidence for a stimulatory effect of MDMA on AVP secretion. However, no female subjects were included. We assessed MDMA effects on AVP system activation and associated changes in plasma and urine osmolality as well as sodium levels in resting healthy subjects with *ad libitum* water intake in a controlled laboratory setting.

MDMA is a substrate of both the serotonin and norepinephrine transporter (7). It enters presynaptic nerve terminals and potently releases serotonin and norepinephrine through the transporter (7). AVP secretion is thought to be regulated by serotonergic (8) and noradrenergic (9) pathways, and these monoamines could act as mediators for the effects of MDMA on the AVP system. The MDMA-induced carrier-mediated release of serotonin and norepinephrine can be reduced by serotonin and norepinephrine transporter inhibitors, respectively (10, 11). We therefore assessed whether blockade of both the serotonin and norepinephrine transporter with duloxetine would prevent potential effects of MDMA on AVP secretion in the present study.

The reliable determination of plasma AVP is problematic. We therefore measured copeptin in addition to AVP levels. Copeptin is the C-terminal part of the AVP precursor preprovasopressin. Copeptin is produced together with AVP in equimolar ratio and exhibits similar kinetics in response to osmotic changes (12–14). In contrast to AVP, copeptin levels remain stable in serum or plasma samples and can easily and reliably be measured (12).

We hypothesized that MDMA would increase AVP and copeptin levels, particularly in women, and that pretreatment with the serotonin-norepinephrine transport inhibitor duloxetine would prevent this effect.

Subjects and Methods

Study subjects

The study was performed in 16 healthy subjects (eight women, eight men). Women were (mean ± SD) 29.0 ± 7.1 yr old. Body weight was 59.0 ± 6.9 kg. Men were 23.3 ± 3.1 yr old. Body weight was 79.5 ± 9.8 kg. Exclusion criteria included age under 18 or over 45 yr, pregnancy (urine pregnancy test before each test session), body mass index below 18.5 or over 25 kg/m^2, personal or family (first-degree relatives) history of psychiatric disorder, regular use of medications, chronic or acute physical illness (normal physical exam, normal electrocardiogram, and standard hematological and chemical blood analyses), smoking, lifetime prevalence of illicit drug use over five times (except for tetrahydrocannabinol), illicit drug use within the last 2 months, and illicit drug use during the study (urine tests before test sessions). Subjects were asked to abstain from excessive alcohol consumption between test sessions and in particular to limit their use to one glass on the day before the test sessions. Subjects abstained from caffeinated beverages on the test days. Female subjects were investigated during the follicular phase (d 2–14) of their menstrual cycle when the reactivity to amphetamines (15) and osmotic sensitivity (16) are expected to be similar to men. All subjects gave their written informed consent before participating in the study, and subjects were paid for participation.

Study procedures

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Basel, Switzerland. The use of MDMA in healthy subjects was authorized by the Swiss Federal Office of Public Health, Bern, Switzerland. The study was registered at www.clinicaltrials.gov (number NCT00990067) with neuroendocrine measures as a secondary outcome. We used a randomized placebo-controlled crossover design with four conditions (placebo-MDMA, placebo-duloxetine, duloxetine-MDMA, and placebo-placebo) in balanced order. Washout periods between sessions lasted 10–14 d. Duloxetine (120 mg) or placebo was administered twice 16 and 4 h before MDMA (125 mg) or placebo, respectively. We assessed plasma and urine osmolality as well as plasma and urine sodium 4 h before and 120 min after MDMA/placebo administration. Plasma levels of copeptin were assessed 4 h before and at 60 and 120 min after MDMA/placebo. Plasma levels of AVP were assessed 4 h before and 120 min after MDMA/placebo. Subjects were not engaged in any physical activity and were resting in hospital beds during the test session. Subjects had a small standardized breakfast at the beginning of each test session. Fluid consumption was not restricted up to a total intake of 2000 ml water during the session and was recorded from 4 h before to 120 min after MDMA/placebo administration when the last hormone measurement was performed. In addition, saline was administered via an iv catheter to keep catheters open for blood sampling at a rate of 100 ml/h from 0–120 min after MDMA/placebo administration. The study design also included additional assessments of subjective and cardiovascular effects, blood drawings for pharmacokinetics, and monitoring of adverse events for 6 h after MDMA/placebo administration as will be described elsewhere (Simmler, L. D., C. M. Hysek, J. Huwyler, M. E. Liechti, unpublished data).

Measurements

Measurements were done in duplicates in a blinded fashion in a single batch. AVP was assessed in EDTA plasma using a RIA (Direct Vasopressin RIA; Bühlmann Laboratories AG, Schönenbuch/Basel, Switzerland). The lower detection limit was 0.82 pmol/liter, and the intraassay precision was 6.0%. Copeptin levels were assessed using an immunoassay (LIA CT-proAVP; B.R.A.H.M.S./ThermoFisher Scientific, Hennigsdorf/Berlin, Germany) as described previously (12) and modified as described previously (14). The lower detection limit was 0.4 pmol/liter, and the intraassay coefficient of variation was less than 5%. Sodium concentrations were measured by indirect potentiometry (Hitachi 917; Roche Diagnostics, Rotkreuz, Switzerland). Osmolality was measured by cryoscopy (Micro Osmometer; Advances Instruments for Switzerland Instruments, Zurich, Switzerland).

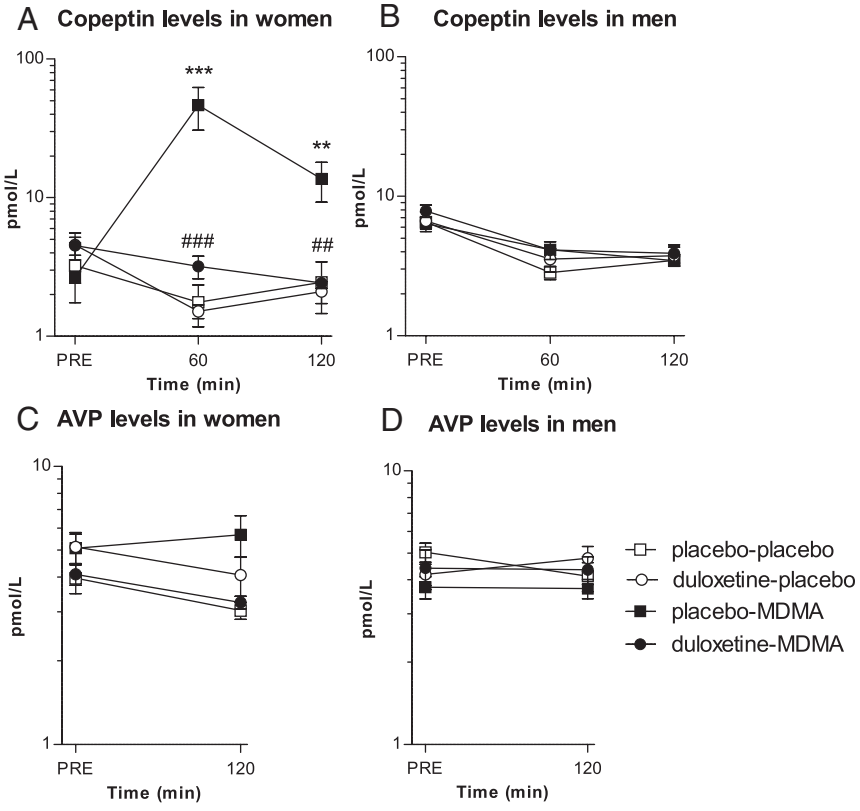

FIG. 1. Mean values ± SEM for plasma levels of copeptin and AVP in eight female and eight male healthy subjects 4 h before (PRE) and 60 and 120 min after MDMA (125 mg) or placebo. A, MDMA significantly increased copeptin levels in women at 60 and 120 min after drug administration compared with placebo. Duloxetine pretreatment prevented the MDMA-induced elevation in circulating copeptin in women. B, MDMA did not alter copeptin levels in men. C, Similar to its effects on MDMA-induced copeptin increases, duloxetine also prevented the nonsignificant increase in AVP at 120 min after MDMA administration in women. D, There were no drug effects on AVP levels in men. **, $P < 0.01$; ***, $P < 0.001$ *vs.* placebo-placebo; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* placebo-MDMA.

Study drugs

(±)MDMA hydrochloride (Lipomed AG, Arlesheim, Switzerland) was obtained from the Swiss Federal Office of Public Health and prepared as gelatin capsules (100 and 25 mg). Identical placebo (lactose) capsules were prepared. MDMA was administered in a single absolute dose of 125 mg. This dose of MDMA corresponds to a typical recreational dose of ecstasy, and comparable doses of MDMA have previously been used in controlled settings. Because MDMA was dosed in an absolute dose of 125 mg, differences in body weight resulted in different weight-adjusted relative MDMA doses of 1.6 ± 0.23 mg/kg (range, 1.4–2.1 mg/kg) in men and 2.1 ± 0.25 mg/kg (range, 1.8–2.5 mg/kg) in women. Duloxetine (Cymbalta; Eli Lilly SA, Vernier, Switzerland) was prepared as 60-mg gelatin capsules, and identically looking placebo (lactose) capsules were similarly prepared.

Statistical analysis

Repeated-measures ANOVA with the factors drug (placebo-placebo, duloxetine-placebo, placebo-MDMA, and duloxetine-MDMA) and time (baseline, 60 min, and 120 min) stratified for sex and followed by pairwise Tukey *post hoc* tests was used to assess differences in the effects of the different drugs. Nonnormally distributed variables were log normalized before the ANOVA. Correlation analyses were performed using Spearman's rank correlations using the total of all values (n = 128). All tests were two tailed, and the significance level was set to $P = 0.05$.

Results

ANOVA on plasma copeptin levels yielded a significant drug × time × sex interaction [$F_{(6,84)} = 3.93$; $P = 0.0017$]. MDMA significantly elevated plasma copeptin levels at 60 min ($P < 0.001$) and at 120 min ($P < 0.01$) compared with placebo in women (Fig. 1A) but not in men (Fig. 1B). The MDMA-induced increase in plasma copeptin in women was prevented by duloxetine pretreatment both at 60 min ($P < 0.001$) and 120 min ($P < 0.01$) (Fig. 1A). A similar trend was observed for AVP levels but drug effects did not reach significance (Fig. 1, C and D). Oral liquid intake varied across drug treatments, but there were no sex differences [main effect of drug: $F_{(3,42)} = 8.62$; $P < 0.001$, no drug × sex interaction]. Oral liquid intake (mean ± SEM) was 612 ± 50 ml after placebo-placebo, 1267 ± 118 ml after duloxetine-placebo ($P < 0.001$ *vs.* placebo-placebo), 1198 ± 130 ml after placebo-MDMA ($P = 0.001$ *vs.* placebo-placebo), and 807 ± 83 ml after duloxetine-MDMA ($P = 0.02$ *vs.* duloxetine-placebo, and $P = 0.051$ *vs.* placebo-MDMA). Urine osmolality decreased significantly over time [main effect of time: $F_{(1,14)} = 62.69$; $P < 0.001$]. Urine osmolality tended to be higher after placebo-MDMA or duloxetine-MDMA compared with placebo-placebo or duloxetine-placebo as evidenced by a near-significant drug × time interaction in the ANOVA [$F_{(3,42)} = 2.70$; $P = 0.058$] (Fig. 2, A and B). A similar trend was observed for urine sodium levels [drug × time interaction: $F_{(3,42)} = 2.33$; $P = 0.088$] (Fig. 2, C and D). There were no significant drug effects on plasma sodium levels or plasma osmolality (Fig. 2, E–H). Circulating copeptin levels correlated with AVP levels (all: $r_s = 0.34$, $P < 0.001$; women: $r_s = 0.53$, $P < 0.001$; men: $r_s = 0.28$, $P < 0.05$]. Copeptin levels were also correlated with plasma and urine osmolality [$r_s = 0.22$; $P < 0.05$ and $r_s = 0.68$; $P < 0.001$, respectively] as well as with plasma and urine sodium [$r_s = 0.18$; $P <$

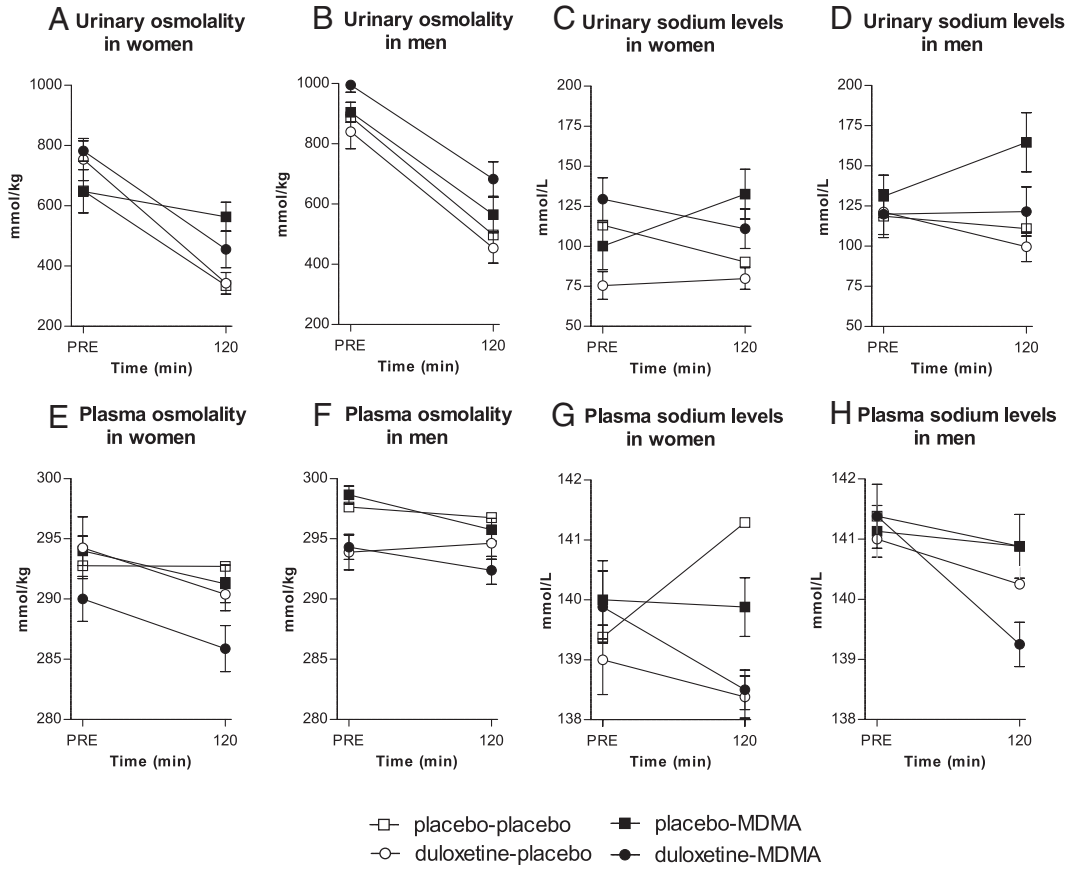

FIG. 2. Mean values ± SEM for sodium and osmolality in urine and plasma in eight female and eight male healthy subjects 4 h before (PRE) and 120 min after MDMA (125 mg) or placebo. The two treatment conditions including MDMA (placebo-MDMA and duloxetine-MDMA) tended to increase both urine osmolality (A and B) and urine sodium levels (C and D) in both sexes. There were no treatment effects on plasma osmolality or plasma sodium levels (E–H).

0.05 and $r_s = 0.28$; $P < 0.01$, respectively]. Baseline copeptin levels were significantly lower in women than men [$F_{(1,14)} = 8.38$; $P = 0.012$]. The relative dose of MDMA (in milligrams per kilogram body weight) did not correlate with the MDMA-induced increase in plasma copeptin within the two sex groups. In the present study, MDMA also produced marked subjective and cardiovascular stimulant effects as will be reported separately elsewhere (Simmler, L. D., C. M. Hysek, J. Huwyler, M. E. Liechti, unpublished data).

Discussion

We found that MDMA increased circulating copeptin, a marker for AVP secretion, in women but not in men. This sex difference in MDMA-induced AVP secretion is in line with the clinical observation that ecstasy-associated hyponatremia is typically reported in female users (3, 4). Other sex differences in the response to MDMA or ecstasy have previously been reported and include increased subjective effects in women compared with men to equal weight-adjusted doses of MDMA (18), more pronounced depression after ecstasy use (19), and a potential increase in serotonergic neurotoxicty in association with long-term use of ecstasy in women (20). The present findings indicate that women may be at increased risk for developing hyponatremia and associated neurotoxicity due to their sex-specific stronger AVP response to MDMA. In addition, the threshold levels of plasma sodium at which neurological complications occur appear to be higher in women than men (21, 22), and woman are more likely than men to die from hyponatremic encephalopathy after surgery (21, 23). Seizures and coma were also more frequently reported in female cases of ecstasy-associated hyponatremia compared with men (3). However, ecstasy-associated hyponatremia may have multiple causes, and MDMA-induced AVP secretion may be only one of several contributing factors. Dry mouth and physical exertion with sweating followed by hyperhydration with electrolyte-free water may all contribute to the development of hyponatremic states in recreational ecstasy users. Even loss of sodium into the gastrointestinal tract has been discussed (24).

The AVP system is activated by factors typically associated with MDMA consumption in a party setting in-

cluding dehydration (12–14), heat (25), and physical activity (12, 26), all of which are potentially increasing the risk of SIADH. Our results indicate that direct activation of the AVP system by MDMA may play a crucial facilitating role in the development of ecstasy-associated SIADH, in particular in women, because we controlled carefully for confounding factors that may increase AVP. Subjects were well hydrated orally and iv and resting comfortably in hospital beds in a temperature-controlled research environment. Of note, our subjects drank more water after MDMA than after placebo administration possibly due to a dry mouth and increased thirst after MDMA administration (18). Fluid consumption would be expected to decrease copeptin secretion (13), counteracting the effects of MDMA. However, copeptin levels were actually increased during the MDMA condition, which further supports the concept that MDMA activated the AVP system via pharmacological stimulation, although we cannot exclude an indirect effect via increased thirst perception (14). Furthermore, urine osmolality and urine sodium levels tended to be higher after MDMA compared with placebo administration despite the increase in oral fluid intake. This finding indicates that MDMA increased renal fluid retention, which is consistent with an elevated secretion of AVP.

The AVP response to MDMA in women was blocked by duloxetine pretreatment. Duloxetine prevents the transporter-mediated release of serotonin and norepinephrine by MDMA. Thus, MDMA-induced AVP secretion appears to be mediated by serotonin and norepinephrine. This clinical finding is in line with preclinical studies indicating a role for central serotonin (8) and norepinephrine (9) systems in AVP secretion. The mediating role of the serotonin system in AVP regulation is also supported by the fact that several serotonergic medications are typically associated with an increased risk of SIADH (22). The precise mechanism of the serotonin/norepinephrine-AVP system interaction is not known. AVP and copeptin are also hypothalamic stress hormones (27, 28), and MDMA is a pharmacological stressor. MDMA activates the hypothalamo-pituitary-adrenal axis and increases plasma corticotropin and cortisol (29, 30). In addition, MDMA increases aldosterone secretion in rats. Cortisol and mineralocorticoids also influence the electrolyte and body fluid balance. We did not assess the role of steroids in the present study. However, steroids increase renal sodium reabsorption and would thereby antagonize AVP effects on plasma osmolality.

In our study, MDMA (125 mg) had no effect on AVP or copeptin plasma levels in male subjects, whereas an earlier study showed an increase in AVP after a lower dose of MDMA (47.5 mg) in eight healthy men (5, 6). This discrepancy is likely due to differences in the study design and setting. Importantly, subjects were free to drink as much as they wanted in our study, and fluid consumption was higher after MDMA than after placebo which could have counteracted any MDMA effects on AVP secretion and even abolished any MDMA effects in men. In addition, our subjects were resting in hospital beds, eliminating any contributing effects of physical activity on AVP secretion. Nevertheless, it is surprising that our comparatively high dose of MDMA did not affect AVP or copeptin secretion despite pronounced subjective and cardiovascular stimulant effects of MDMA in the same subjects (Simmler, L. D., C. M. Hysek, J. Huwyler, M. E. Liechti, unpublished data). Interestingly, similar inconsistencies are seen in the clinical reports on ecstasy-associated hyponatremia. Hyponatremia was found in 55 (52.4%) of 105 women and 18 (21.7%) of 83 men in ecstasy exposures reported to the California Poison Control System (3). However, other reports indicate that hyponatremia is a relatively rare complication of ecstasy use. Ecstasy-associated hyponatremia was observed in only two (5%) of 40 monointoxications (31) or was not reported (32) according to other poison center studies. Hyponatremia was also a rare medical complication according to a series of intoxication cases presenting to emergency rooms (17, 33, 34). Taken together, the available data point toward an important role of additional contributing personal (sex, menstrual phase, and genetic factors) and/or environmental (heat and hydration) factors that may contribute and modulate the effects of MDMA on AVP secretion and osmotic regulation.

Our study has several limitations. The study sample size is relatively small. Only single doses of MDMA and duloxetine were used. However, the doses were selected in the upper dose range and produced pronounced effects on a variety of outcomes. Importantly, the absolute dose of MDMA was the same in both sexes and was not adjusted for body weight, resulting in higher relative doses of MDMA per kilogram of body weight in women compared with men. Thus, we cannot exclude that the observed sex difference was in fact a dose effect with women receiving higher relative doses of MDMA than men. However, relative MDMA doses did not correlate with MDMA-induced changes in copeptin levels within the male and female groups, supporting the view that our finding represents a true sex difference and not a dose effect. Furthermore, fluid consumption was different across treatment conditions, which may have counteracted effects of MDMA on AVP secretion because subjects consumed more liquids after MDMA than after placebo administration. Finally, urine osmolality and associated AVP system activation was higher in men than women at the beginning

of the study, which may have differentially affected the response to MDMA.

With regard to the validity of the outcome measures, we documented a correlation of plasma AVP and copeptin, confirming previous studies (12, 14). In addition, copeptin plasma concentrations also weakly correlated with plasma and urine osmolalities as expected based on osmoregulation and as previously documented in hypo-, iso-, and hyperosmolar states in healthy subjects (14). We also confirmed the previously reported sex differences in basal plasma copeptin concentration (12, 13).

In conclusion, we found that MDMA increased copeptin plasma levels reflecting AVP system stimulation in women but not in men. The finding is consistent with an increased risk for the development of hyponatremia and associated complications after recreational ecstasy use in women compared with men. AVP system activation by MDMA is likely due to the serotonin- and norepinephrine-releasing properties of MDMA.

Acknowledgments

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Disclosure Summary: The authors declare no conflict of interest.

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Chapter 3: In vitro studies relevant for the pharmacological treatment of stimulant addiction

Stimulant dependence comes along with the action of the questioned amphetamine-type drugs at dopaminergic targets. In paper 3 we investigated the effects of different treatments on stimulant-induced DA release, which is directly responsible for the addictive properties of a drug. Namely, we assessed the potencies and suitability of the antidepressant and smoking-cessation aid bupropion, the ADHS medication methylphenidate, and the abused but highly potent DA uptake inhibitor MDPV to inhibit methamphetamine-induced DA release in vitro with relevance for methamphetamine dependence.

Content of chapter 3:

Paper 3: Bupropion, methylphenidate, and 3,4-methylenedioxypyrovalerone antagonize methamphetamine-induced efflux of dopamine according to their potencies as dopamine uptake inhibitors: implications for the treatment of methamphetamine dependence

Paper 3

Bupropion, Methylphenidate, and 3,4-Methylenedioxypyrovalerone Antagonize Methamphetamine-Induced Efflux of Dopamine According to their Potencies as Dopamine Uptake Inhibitors: Implications for the Treatment of Methamphetamine Dependence

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SHORT REPORT Open Access

Bupropion, methylphenidate, and 3,4-methylenedioxypyrovalerone antagonize methamphetamine-induced efflux of dopamine according to their potencies as dopamine uptake inhibitors: implications for the treatment of methamphetamine dependence

Linda D Simmler, Rebecca Wandeler and Matthias E Liechti*

Abstract

Background: Methamphetamine-abuse is a worldwide health problem for which no effective therapy is available. Inhibition of methamphetamine-induced transporter-mediated dopamine (DA) release could be a useful approach to treat methamphetamine-addiction. We assessed the potencies of bupropion, methylphenidate, and 3,4-methylenedioxypyrovalerone (MDPV) to block DA uptake or to inhibit methamphetamine-induced DA release in HEK-293 cells expressing the human DA transporter.

Findings: Bupropion, methylphenidate, and MDPV inhibited methamphetamine-induced DA release with relative potencies corresponding to their potencies to block DA uptake (potency ranks: MDPV > methylphenidate > bupropion).

Conclusions: Bupropion and methylphenidate antagonize the effects of methamphetamine in vitro and may be potential candidates for the treatment of stimulant addiction. However, drugs that very potently antagonize the effect of methamphetamine are likely to also exhibit considerable abuse liability (MDPV > methylphenidate > bupropion).

Keywords: Methamphetamine, Addiction, Dopamine, Dopamine transporter, Bupropion, Methylphenidate, MDPV

Findings

Background

Methamphetamine dependence is a major public health problem. Currently, no medical treatments are approved for stimulant dependence indicating the need to explore potential candidates [1]. Methamphetamine releases dopamine (DA) via the DA transporter (DAT) [2,3]. DA is thought to mediate the reinforcing effects of psychostimulants, which lead to drug dependence [4,5]. Blocking the pronounced release of DA by methamphetamine may therefore be an interesting therapeutic option for the treatment of methamphetamine dependence [1]. Bupropion and methylphenidate are DA uptake inhibitors that interact with the same pharmacological target as methamphetamine [6-11]. Bupropion is used as an antidepressant and smoking cessation aid [7,9]. Methylphenidate is effectively used in the treatment of attention-deficit/hyperactivity disorder [12,13]. In addition, small clinical studies indicated promising beneficial effects for both medications in methamphetamine dependence [1]. Bupropion reduced the acute subjective effects of methamphetamine in a laboratory study [14] and methamphetamine use in dependent patients with moderate drug use [15-18]. Methylphenidate reduced amphetamine use in dependent patients [19] and it is now being investigated in methamphetamine-addiction (clinicaltrials.gov: NCT01044238). Bupropion also reduced methamphetamine self-administration in rats [20] or rhesus

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monkeys [21]. In contrast, methylphenidate did not affect methamphetamine self-administration in rhesus monkeys [21].

The precise pharmacological mechanism of action of bupropion and methylphenidate with regard to their therapeutic effects in methamphetamine dependent patients is not known. Dopamine is thought to contribute to the drug-high and euphoria produced by psychostimulants and mediates the addictive properties of drugs of abuse [4,22]. Amphetamines reverse the transport of DA through the DAT and this effect is thought to play a key role in the addictive potential of amphetamines [5]. The DA uptake inhibitors bupropion and methylphenidate may therefore prevent methamphetamine from interacting with the DAT to release DA, and such an effect would antagonize effects of methamphetamine. Several DA uptake inhibitors have previously been shown to prevent DAT-mediated release of DA by amphetamines in vitro. For example, bupropion and methylphenidate [23] as well as GBR12909 [3] inhibited DAT-mediated amphetamine- or methamphetamine induced DA release from rat synaptosomes. In HEK-293 cells expressing human DAT, methylphenidate inhibited DA efflux induced by methamphetamine [24]. These and similar data suggest that bupropion and methylphenidate block the interaction of methamphetamine with the DAT to release DA and thereby act as antagonists of amphetamine-like drugs.

The aim of the present study was to test and compare the effects of bupropion and methylphenidate on methamphetamine-induced DA efflux in HEK-293 cells expressing human DAT in vitro. Bupropion and methylphenidate were selected because of their availability as licensed medications and the clinical data described above. We also included 3,4-methylenedioxypyrovalerone (MDPV) into the study because it has been shown to be a very potent DAT inhibitor [10,25].

We hypothesized that 1) the DA uptake blockers would prevent methamphetamine-induced DA release and 2) the potencies of the drugs to inhibit methamphetamine-induced DA release would correspond to their potencies to block DA uptake.

Methods

Drugs

(±)-Bupropion hydrochloride was from Toronto Research Chemicals (North York, Canada), d-methamphetamine, (±)-methylphenidate, and (±)-MDPV were supplied as hydrochloride salts by Lipomed (Arlesheim, Switzerland).

Inhibition of DA uptake

The potencies of the drugs to inhibit the DAT were evaluated as previously described [26] in HEK-293 cells (Invitrogen, Zug, Switzerland) stably transfected with the human DAT [8].

Inhibition of methamphetamine-induced DA release

We performed DA transporter mediated release experiments as previously published [25] with slight modification. In brief, HEK-293 cells expressing the human DAT as stated above were cultured in 48 well-plates. Cells were filled with ^{3}H-DA, washed, and incubated with 250 μL buffer containing the drug alone or in combinations. Drug combinations were 10 μM of methamphetamine with bupropion, methamphetamine, or MDPV in different concentrations. DA release was stopped after 15 min by removing the release buffer from the cells. To quantify the DA release we determined the radioactivity in the cells after another wash step. The residual radioactivity in the cells after methamphetamine alone defined 100% DA release. Baseline (0% release) was defined as the radioactivity remaining in the cells treated with bupropion, methylphenidate, or MDPV alone at the highest concentration used.

Results

Inhibition of DA uptake

Bupropion, methylphenidate, and MDPV inhibited the uptake of DA. MDPV was the most potent DAT inhibitor followed by methylphenidate and bupropion. Methamphetamine blocked DA uptake with similar potency to bupropion (Figure 1).

Inhibition of methamphetamine-induced DA release

Methamphetamine released DA with a potency (EC_{50}) of 1.56 μM (0.9 μM-2.8 μM, 95% CI) as shown previously [25]. DA release induced with 10 μM methamphetamine was inhibited concentration-dependently by bupropion, methylphenidate, and MDPV (Figure 2). MDPV was the most potent inhibitor of the methamphetamine-induced DA release followed by methylphenidate and bupropion (Figure 2). The IC_{50} values are shown in Table 1. The potencies (IC_{50} values) of the drugs to block DA release correlated highly with the potencies to block DA uptake (Figure 3) as confirmed by a correlation coefficient of >0.99, $p < 0.05$.

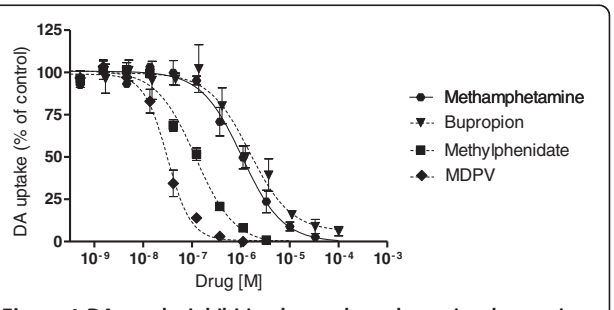

Figure 1 DA uptake inhibition by methamphetamine, bupropion, methylphenidate, and MDPV. IC_{50} values are shown in Table 1. The data are expressed as mean ± SEM of 3–4 independent experiments.

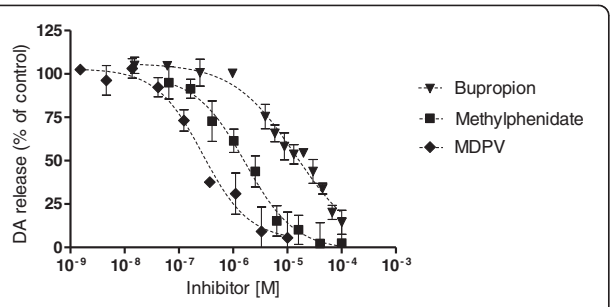

Figure 2 Methamphetamine-induced DA release is inhibited by bupropion, methylphenidate and MDPV. The corresponding IC_{50} values are shown in Table 1. DA release was induced with 10 μM methamphetamine (100% release) and blocked with different concentration of the inhibitors bupropion, methylphenidate or MDPV. Baseline (0% release) was defined as the radioactivity remaining in the cells treated with bupropion, methylphenidate, or MDPV alone. The data are expressed as mean ± SEM of 3–4 independent experiments.

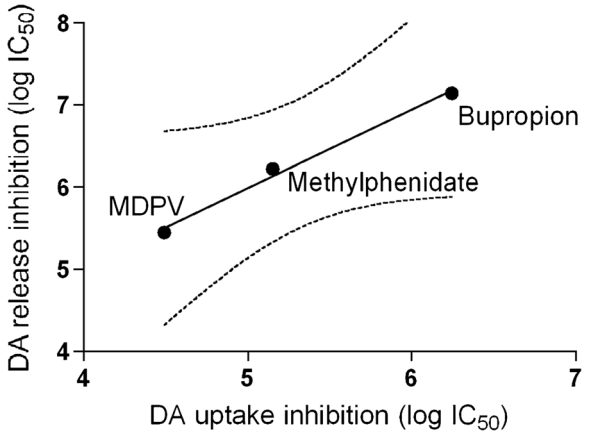

Figure 3 The potencies of the drugs (log IC_{50} values) to inhibit DA uptake correlate linearly with their potencies to inhibit methamphetamine-induced DA release (correlation coefficient > 0.99, $p < 0.05$, regression line and 95% confidence intervals).

Discussion

In the present study, the DA uptake inhibitor bupropion inhibited DA release induced by methamphetamine. This mechanism might underlie the reduction in the methamphetamine-induced subjective drug high by bupropion pretreatment documented in a clinical laboratory study [14] and the reduced methamphetamine consumption in drug users treated with bupropion [15-18]. Methylphenidate also blocked the methamphetamine-induced DA release similar to bupropion and this effect may also antagonize the rewarding effects of methamphetamine and its use in dependent patients. In fact, methylphenidate showed beneficial effects in amphetamine dependent patients [19] and is being investigated for the treatment of methamphetamine addiction (clinicaltrials.gov: NCT01044238). Thus, inhibition of DA release might be a pharmacological mechanism how DA uptake inhibitors reduced the subjective stimulant drug effects or drug consumption in the clinical studies noted above. In addition, methylphenidate and bupropion also increase DA levels and therefore both drugs may also act as substitution treatments for methamphetamine use. In the present study we also included the very potent DA uptake inhibitor MDPV to explore how the potency of a drug as DA uptake inhibitor relates to its potency to antagonize the pharmacological effect of methamphetamine. MDPV blocked methamphetamine-induced DA release with high potency reflecting its high potency as an uptake inhibitor. We found that the potencies of the drugs to block methamphetamine-induced DA release correlated closely and significantly with their potencies to act as DA uptake inhibitors. The finding suggests that the more potent a drug antagonizes the DA release produced by methamphetamine the more potently it also blocks DA uptake. This finding may have important clinical implications regarding the abuse liability of potential antagonist treatments for methamphetamine dependence. With regard to the drugs tested in the present study, the antidepressant bupropion is a low-potency DA transporter inhibitor and it is considered a drug that does not produce relevant euphoria nor addiction [27,28]. Methylphenidate is an intermediate-potency DA transporter inhibitor and is known to produce euphoria at higher doses [29,30] and to have a relevant abuse potential [31,32]. The cathinone MDPV is a high potency DA transporter inhibitor and street designer drug ("super coke", "research chemical", "bath salt") with high addiction potential similar to the DA releaser methamphetamine [25,33-35]. Our findings indicate that drugs that potently and effectively antagonize the effect of methamphetamine are likely to exhibit high abuse liability themselves because they block DA uptake. In fact, the potency of amphetamine-type stimulants to block DA uptake has been shown to correlate with the doses used by humans [25]. Furthermore, potent DA transport uptake inhibition is sufficient to produce addiction because cocaine and MDPV only block DA uptake and do not induce DA release as methamphetamine [5,25]. It is therefore questionable whether there are any compounds that do not activate the

Table 1 Potencies of drugs to block DA uptake or methamphetamine-induced DA release

	DA uptake	Methamphetamine-induced DA release
	IC_{50} (μM) (95% CI)	IC_{50} (μM) (95% CI)
Methamphetamine	1.05 (0.7-1.5)	-
Bupropion	1.76 (1.1-2.8)	14.2 (9.7-21)
Methylphenidate	0.14 (0.1-0.2)	1.67 (0.7-4.0)
MDPV	0.031(0.03-0.04)	0.28 (0.1-0.6)

Values are means of 3–4 independent experiments and 95% confidence intervals (CI).

DA system and lack abuse liability but effectively prevent methamphetamine from interacting with DAT. On the other hand, abuse liability of medications can be reduced by using extended-release drug formulations.

Methamphetamine also has additional effects on the DA system (e.g., on monoamine oxidase and the vesicular monoamine transporter), which were not studied here. These effects of methamphetamine take place within the cells and are likely prevented by DAT inhibitors [36] that block methamphetamine transport into the cell. Methamphetamine also releases norepinephrine [2,3] and norepinephrine is thought to contribute to the acute effects of amphetamine-type drugs [3,37-39]. MDPV [25] and methylphenidate [6,40], and to a lower extent bupropion [7,11], block the norepinephrine transporter and these drugs could also block methamphetamine-induced norepinephrine release. We did not address potential drug interactions at the norepinephrine transporter because in contrast to DA, norepinephrine is not generally thought to be a major mediator of the addictive properties of psychostimulants. However, interactions at the norepinephrine transporter could be expected to contribute to any therapeutic effects of the drugs tested in the present study. Finally, it should be noted that we assessed only a small number of DAT inhibitors. However, the drugs were selected to cover a wide range of DAT inhibition potencies including also the very potent DAT inhibitor MDPV.

Conclusion

Our in vitro studies and the limited clinical data indicate that the low- and intermediate-potency DA uptake inhibitors bupropion and methylphenidate may be potential candidates as treatments of amphetamine-type stimulant dependence [1] due to their property to inhibit methamphetamine-induced DA efflux. Their clinical efficacy needs further confirmation.

Availability of supporting data

The data supporting the results of this article are included within the article. This work was supported by the Swiss National Science Foundation (grant no. 32323B_144996). Publication costs are supported by the Neurex network (www.neurex.org).

Abbreviations

DA: Dopamine; DAT: Dopamine transporter; MDPV: 3,4-methylenedioxypyrovalerone.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LDS and MEL designed this study and wrote the manuscript. RW and LDS performed the experiments. All authors analyzed the data. All authors have read and approved the final manuscript.

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Discussion

We assessed the pharmacology of amphetamine-type drugs in vitro and, using MDMA, also in humans. We found that these novel designer drugs overall resemble long known psychostimulant drugs like MDMA, methamphetamine, or cocaine. However, there is considerable variability in the pharmacological properties within cathinone designer drugs. We therefore classified the assessed novel designer drugs according to their pharmacology, which should also facilitate the prediction of their psychotropic effects, expected medical complications, and presumed addictive potential in humans. Currently abused novel designer drugs range from highly potent dopaminergic and noradrenergic drugs (e.g., MDPV) to selective serotonergic drugs (e.g., methedrone), thus displaying a wide range of potential effects in humans. We demonstrated that in vitro pharmacological profiling was a useful tool to gain a first and fast overview on the pharmacology of newly emerging designer drugs with action on the monoamine systems. We also highlighted the essentiality of clinical laboratory studies in humans to clarify the role of the different monoaminergic neurotransmitters and receptors in the psychotropic and clinical toxicological effects of these recreationally used drugs. Translating the in vitro to the clinical findings and back, we gained knowledge and certainty in the interpretation of pharmacological effects of amphetamine-type drugs. With the understanding of the drugs' pharmacology, we took the next step to explore ways to combat drug-related problems such as stimulant dependence by pharmacological antagonism.

Methodological problems and limitations

During the establishment of the pharmacological in vitro assays, we were facing several methodological problems. I address here the most crucial issues.

As shortly mentioned in the methods of paper 1a, in our hands, a pure uptake inhibitor induced slight "transporter-mediated" monoamine release from monoamine-preloaded cells. For example, mazindol and citalopram reduced radioactivity remaining in cells by ≤ 20% in HEK DAT and HEK SERT cells, respectively (see paper 1a). Some other research groups did not explicitly report this so-called apparent release both in HEK cell lines [1] and in synaptosomes [2]. However, there is an exemplary publication

that explains the cause of this apparent monoamine release by pure uptake inhibitors as methodological artifact. In their experiments with superfused HEK SERT cells, pure 5-HT uptake inhibitors like fluoxetine induced marked 5-HT release. This release was not as effective than the one induced by a substrate-type releaser such as *para*-chloroamphetamine. From their in vitro studies assessing the cause of this phenomenon, the authors concluded that intracellular 5-HT must penetrate the plasma membrane in a transporter-unspecific way, but in presence of a highly potent uptake blocker like fluoxetine there is no subsequent transporter-mediated 5-HT reuptake into the cell. This causes the nonspecific or apparent release observed when calculating monoamine efflux from a non-blocking vehicle control [3]. Scholze et al. proposed to use 1-methyl-4-phenylpyridinium (MPP^+) as a substitute for 5-HT to circumvent this apparent release phenomenon. However, we and others decided to continue our experiments with the physiological substrates because MPP^+ and 5-HT, NE, or DA do not have identical transport characteristics [3-5]. Considering apparent release in the experimental setting by subtraction or statistical analysis, we could keep on working with the natural neurotransmitters.

A further difficulty in our methodology should be mentioned here. We preloaded cells with neurotransmitters prior to release-induction to ensure an intracellular reservoir of monoamines that could be released, thus modeling a transmitter-loaded presynapse of a neuron. For 5-HT release in HEK SERT cells, loading the cells with 10 nM 5-HT was sufficient for effective release. However, for DA and NE release, 1 µM DA and 10 µM NE were required to obtain effective monoamine release. This was shown for DA before [6], but others succeeded using low DA or NE concentrations [1, 7, 8].

Amphetamines that induce monoamine release are generally thought to be substrates of the respective monoamine transporters [9]. Since the release assay remained to be difficult, we also tried to assess whether substances were transporter substrates to classify a drug as substrate-type releaser or as non-releaser. Thus, we quantified intracellular drugs after a 15 min period of uptake at 37 °C and on ice, with or without uptake inhibitors present. Unfortunately these attempts remained unsuccessful, as data were difficult to interpret due to high variability. It also seemed that uptake on ice could still take place, which would contradict the claim of active substrate transport [10]. Moreover, we could not distinguish between a releasing- and a non-releasing substance, as all drugs were transported to a certain extent, probably due to their

lipophilicity. Thus, we remained unsuccessful in quantifying substrate transport, but experimental optimizations (e.g. reduction of variability and passive transport) could indeed lead to interesting information on drugs' transport characteristics and toxicological potential. These experiments are mentioned here for completeness but are not described in the result section where only more conclusive published or publishable data was presented.

It should be noted here that attempts to characterize substrate uptake into human cells or synaptosomes and monoamine release experiments are in general considered methodologically complex and less reliable and valid than commonly used monoamine uptake assays. Release may therefore best be studied using in vivo microdialysis in freely moving rodents. However, this approach is very resource-consuming and can only be used to evaluate a selection of compounds as recently done by other research groups [11, 12].

Using transfected cells as model to assess drugs' pharmacology, we took advantage of the possibility to work with human transporters. This is not possible if synaptosomes were used, since they are derived from non-humans such as rhesus monkeys [2] or mice [13]. However, working with cells transfected with one respective gene, we do not take into account that genetic variants of SERT, DAT, and NET exist and that these variants might exert different effects as response on drug action [14-18]. To account for different geno- and phenotypes would, however, exceed this thesis' possibilities. In addition, in transfected human cells only the target transporter or receptor of interest is evaluated in isolation whereas synaptosomes contain all molecules present in the synaptic membrane. We consider the in vitro models with human transporters and receptors an excellent starting point to rapidly screen for the pharmacological properties of newly emerging substances. These experiments can then be complemented by in vivo tests in rodents (locomotor behavior, cardiovascular stimulation, telemetry with body temperature recordings, and microdialysis) for selected compounds of interest, ultimately also using clinical studies for a subset of compounds that are more widely used recreationally.

In this thesis, we hardly broach the issue of stereochemistry in the assessed drugs. All of the assessed substances exist as two enantiomers. For MDMA, amphetamine, and methamphetamine it is well known that their pharmacology and even pharmacokinetics are stereoselective [1, 2, 19]. Novel designer drugs like cathinone-

derivatives are mainly commercially available as racemates only, therefore we worked, with the exception of amphetamine and methamphetamine, with the respective racemic mixture.

Some of our studies, namely paper 1a, 1b and paper 2b in this thesis, provide binding affinity constants derived with assays which quantify the ability of a drug to displace a radiolabeled ligand from the binding site of the target. We admit that the significance of this information is limited if the radiolabeled ligand and the substance to assess do not share the same binding site and property. A close look at our data reveals that affinity data for transporter substrates do not correlate with their potency to inhibit the transporter. MDMA, for example, inhibits NE and 5-HT uptake with similar potencies than cocaine, but its binding is approximately 10 times weaker compared to cocaine (see paper 1a). This is a common phenomenon for binding studies using ligands that are also transporter substrates because transport of the substrate alters the apparent binding affinity [20-22]. Furthermore, the non-releaser cocaine and the substrate-type releaser MDMA do not share the same binding site at the transporters, which was shown nicely for SERT by the Blakely group [23]. Obviously, if a substance is a substrate-type releaser, its binding affinities assessed by the described displacement assay will never be representative. In fact, we could even use the discrepancy of the binding affinities and uptake inhibition potencies as marker for the drug being a substrate and thus a monoamine releaser.

Differentiation of releasers and non-releasers in relation to psycho-stimulant effects in humans

Throughout my PhD thesis, I distinguished monoamine-releasing drugs from pure monoamine uptake inhibitors, either in the characterization of their pharmacology or in the interpretation of their impact on humans. We came across the fact that MDPV exerts extremely pronounced psychostimulant effects in humans [24-26], but found it to be a pure uptake inhibitor. Before this finding, we have speculated that the releasing property of a drug might result in more potent effects compared to a non-releasing drug. However, whether a psychostimulant releases monoamines (such as MDMA or amphetamine) or acts primarily as an uptake inhibitor (such as cocaine or MDPV) may not be critically relevant with regard to the psychostimulant and addictive properties of a drug in vivo. With MDPV and cocaine we know two highly

effective and potent psychotropic but non-releasing drugs [27, 28], and also methylphenidate is a pure uptake inhibitor, which is frequently abused due to its stimulating properties [29]. On the other hand, at least with regard to the 5-HT and NE, an uptake inhibitor abolishes psychostimulant effects of the 5-HT/NE-releaser MDMA in humans, as shown in paper 2a and 2b as well as by Liechti et al. [30]. Thus, net monoamine accumulation should be lower in presence of a non-releasing drug compared to a releasing drug. For example, 5-HT concentrations in the synaptic cleft would not be increased by citalopram to the same extent as it would be by MDMA, although citalopram inhibits 5-HT uptake at SERT with higher potency than MDMA [2]. This was indeed observed with DA in two studies comparing extracellular DA concentrations measured by microdialysis. Cocaine provoked lower extracellular DA concentrations than equivalent amphetamine doses [31, 32]. In contrast, another study by Maisonneuve et al. claimed no differences between net DA concentrations after cocaine or amphetamine administration [33]. Due to the underrepresentation of studies comparing net extracellular neurotransmitter levels induced by non-releasing and releasing drugs, I hereby cannot draw final conclusions on the relevance to distinguish between a non-releasing and releasing psychoactive drug for its psychostimulant effects.

The effects of a psychostimulant on the DA and NE systems appear to be the critical determinant of the psychostimulant effectiveness of a drug, regardless of its releasing or non-releasing properties. In fact, the DAT and NET but not the SERT inhibition potency of the psychotropic drugs investigated previously [2] or in this work (paper 1a) correlated with the doses to induce psychostimulant effects in humans. Furthermore, release of DA, but also pure DA uptake blockade, is related to increased risk for addiction [34]. Rothman suggested over 20 years ago that there are uptake inhibitors like cocaine which produce addiction and euphoria and uptake inhibitors like bupropion which are equally potent DAT blockers, but do not have addictive or psychostimulant effects [35]. These kind of non-addictive and non-euphoriant uptake inhibitors could indeed be used as pharmacological treatment against addiction. We should also consider that dosing and pharmacokinetics are highly critical additional determinants of the abuse and euphoriant potential of psychostimulants. DA uptake inhibitors like methylphenidate may produce very pronounced and identical

psychostimulation and feelings of drug high to cocaine when administered in high doses, intravenously, or by the nasal route [29].

Finally, we should always consider the increased toxic potential of a substrate-type releasing drug due to its intracellular accumulation [36]. A pure uptake-inhibiting drug could even prevent this toxicity by inhibiting the transporter-mediated uptake of the toxic drug into the presynaptic neuron [10].

Relevance of our findings for medical care

For our society the main benefit from our research is the pharmacological characterization of so far poorly evaluated but recreationally used drugs of abuse. Knowing about the molecular pharmacology of these designer drugs, we can predict some of the acute clinical effects and medical complications. We can thereby advise physicians on the expected features of intoxications and which emergency treatment would be appropriate based on pharmacological considerations, in accordance e.g. to the recently reported recommendations by Mas-Morey et al. [37]. Moreover, we can estimate the addictive potential of a drug based on their dopaminergic versus serotonergic properties.

As we saw in our study presented in paper 2c and in the work by others [38, 39], MDMA use is associated with SIADH and hyponatremia due to its 5-HT releasing properties. Thus, we expect all 5-HT releasing drugs to potentially produce SIADH. A recent report by Boulanger-Gobeil et al. [40] is in line with this interpretation. They found hyponatremia, resulting from SIADH, in a case that was exposed to methylone and ethcathinone. Indeed, we showed methylone to be a 5-HT releasing amphetamine-derivative (see paper 1a). For all intoxications with 5-HT releasing drugs, we would therefore advise emergency care personnel to consider hyponatremia if intoxication cases present with confusion, seizures or even comma. They should treat these intoxications primarily with water restriction [41].

More common than hyponatremia-related toxicity are signs and symptoms associated with sympathomimetic overstimulation such as hypertension, tachycardia, hyperthermia, and agitation due to psychostimulant use [36, 42]. Indirect agonism at the adrenoreceptors α_1 and $\beta_{1\text{-}3}$ are responsible for cardiostimulant effects of NE uptake inhibiting and/or releasing drugs [36, 43-45]. Cardiostimulation results in high blood pressure, increased heart rate, and increased body temperature [36, 43].

Drug-induced body temperature elevation is triggered by physical activity in hot environments such as dance parties [46, 47]. This kind of hyperthermia can be life-threatening because it might result in multi-organ failure [47, 48]. The clinical picture of amphetamine-associated sympathomimetic overstimulation including hyperthermia is typically seen with drugs stimulating the noradrenergic system [36, 49]. In our in vitro profiling in chapter 1, all novel designer drugs inhibited NE uptake considerably. We therefore expect possible sympathomimetic intoxications with all assessed designer drugs. Medical personnel should be aware of treating these cases of sympathomimetic intoxication due to consumption of new designer drugs analog to intoxications with amphetamine, cocaine, and MDMA. Typical pharmacological intervention implies sedation with benzodiazepines [27, 37, 48]. Beta-blockers or α_1-β-blockers are not commonly used in emergency care treatment of stimulant intoxications. One advises against the use of β-blockers due to observed unopposed α_1-activation in response to cocaine which may result in enhanced vasoconstriction and tachycardia [50]. In MDMA, the β-blocker pindolol did not worsen drug-induced cardiovascular effects. It might be useful to improve MDMA-induced tachycardia, but not hypertension [51]. However, we showed that the α_1-β-blocker carvedilol reduces MDMA-induced cardiostimulation and more importantly also the thermogenic effects of MDMA [44]. Apart from benzodiazepines, an α_1-β-blocker could therefore also be useful in stimulant intoxications in particular in cases with severe hyperthermia that do not adequately respond to fluid administration and sedation.

Amphetamine-induced psychotic symptoms such as paranoia and hallucinations derive mostly from DA release [36]. Among novel designer drugs, cathinone-derivatives are characterized by pronounced activity at dopaminergic neurons (see chapter 1). We expect psychotic symptoms to arise from cathinones-use, which indeed is reported in the literature [26]. In severe cases, antagonism of psychosis with neuroleptics may be indicated, but benzodiazepines should be used first [27, 52].

Within the scope of this thesis, we strongly focused on the medical risks that an amphetamine-type drug bears when misused for recreational purpose. However, I want to remind here that there are indeed positive medical aspects of some amphetamine-type drugs. Most notably, amphetamine and methylphenidate are effective and widely prescribed in the treatment of attention deficit hyperactivity

disorder [53]. Before illegalization, the entactogenic MDMA was widely used in psychotherapy to facilitate the therapist's access to the patient. There is also a renewed research focus on the use of MDMA as adjunct to psychotherapy in the treatment of post-traumatic stress disorders (PTSD) [54, 55]. Recent clinical studies showed MDMA's beneficial and catalytic effects for recovery from PTSD [56-58].

Outlook

Accessible knowledge about the designer substances currently abused and their pharmacology and clinical toxicology is highly valuable. Thus, we keep on profiling the pharmacology of novel designer drugs in vitro. In collaboration with emergency care doctors and medical analysts, we will also collect and report cases requiring emergency care due to designer drug intoxications. These efforts will further improve the understanding of recently emerged drugs of abuse and support medical care personal to treat such intoxications, as well as to help health authorities in their decisions to schedule new drugs.

Wherever the pharmacology of novel designer drugs is unknown, their toxic effects are unknown either and remain an object of concern. Since we now hold a comprehensive set of frequently abused drugs in our laboratory, we should take the chance and study their neurotoxicological potential as well. We could assess the implications of our designer drugs on mitochondrial dysfunction in vitro. After incubation with liver microsomes [59], we could also assess the toxic effects of their metabolites, which probably bear even higher toxicological potentials than the mother substances [60]. One could also study the in vitro potencies to inhibit enzymes involved in neurotransmitter synthesis and metabolism in particular with those drugs that are substrate-type releasers and thus transported into the cells.

One should also take advantage of the mechanistic understanding of the drugs' pharmacology and the contextual neuronal functions. In vitro, the direct antagonism of psychostimulant drugs' targets is shown by us in paper 3 and by others [2, 61, 62], but one should intensify the investigations on the benefits of this antagonism in humans [63]. We need more data from clinical studies assessing for example the outcome from treatment with DAT inhibitors on stimulant dependence. Besides of direct interaction with stimulant targets, we also propose to further investigate indirect pharmacological modulation. Animal experiments showed the involvement of the

trace-amine associated receptor 1 in drug effects [13, 64]. We propose to keep on investigating the possibility to modulate effects of drugs of abuse via the pharmacological activation of this receptor. However, more mechanistic understanding of this pathway is needed before addressing this objective in humans.

Irrespective of the different objectives proposed here for further investigations, the continuous use of a wide variety of psychostimulants points towards the essentiality of doing research on currently abused drugs. Manhood will never stop changing its perception by the ingestion of effective substances. Is this not reason enough for all efforts to understand the negative and positive consequences of drugs?

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Teaching Experiences

Supervision of master's theses:

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Publication List

1. Hysek CM, Fink A, **Simmler LD**, Donzelli M, Grouzmann E, Liechti ME: α1 Adrenergic receptors contribute to the acute effects of MDMA in humans. *J Clin Psychopharmacol in press.*
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