Measuring inhibition of monoamine reuptake transporters by new psychoactive substances (NPS) in real-time using a high-throughput, fluorescence-based assay

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ABSTRACT

The prevalence and use of new psychoactive substances (NPS) is increasing and currently over 600 NPS exist. Many illicit drugs and NPS increase brain monoamine levels by inhibition and/or reversal of monoamine re-uptake transporters (DAT, NET and SERT). This is often investigated using labor-intensive, radiometric endpoint measurements.

We investigated the applicability of a novel and innovative assay that is based on a fluorescent monoamine mimicking substrate. DAT, NET or SERT-expressing human embryonic kidney (HEK293) cells were exposed to common drugs (cocaine, DL-amphetamine or MDMA), NPS (4-fluoroamphetamine, PMMA, α-PVP, 5-APB, 2C-B, 25B-NBOMe, 25I-NBOMe or methoxetamine) or the antidepressant fluoxetine.

We demonstrate that this fluorescent microplate reader-based assay detects inhibition of different transporters by various drugs and discriminates between drugs. Most IC_{50} values were in line with previous results from radiometric assays and within estimated human brain concentrations. However, phenethylamines showed higher IC_{50} values on hSERT, possibly due to experimental differences.

Compared to radiometric assays, this high-throughput fluorescent assay is uncomplicated, can measure at physiological conditions, requires no specific facilities and allows for kinetic measurements, enabling detection of transient effects. This assay is therefore a good alternative for radiometric assays to investigate effects of illicit drugs and NPS on monoamine reuptake transporters.

1. Introduction

The use of illicit drugs is high and 5% of the population worldwide used an illicit drug in the last year. Frequently used drugs include cocaine, amphetamine and 3,4-methylenedioxy-N-methylamphetamine (MDMA). While the prevalence of use of these common drugs is decreasing, the use of new psychoactive substances (NPS) is steadily increasing (UNODC, 2016). A European survey conducted amongst young European adults (15–24 year old) reported a lifetime prevalence for NPS use of 8% (Flash Eurobarometer 401, 2014).

In the Netherlands, the Drugs Information and Monitoring System (DIMS) offers a drug testing service to drug users. Data showed that, although NPS are also sold as common illicit drugs, the use of NPS as a drug of choice is increasing. The most frequently detected NPS in drug

samples were 2,5-dimethoxyphenethylamine (2C-B), 4-fluor-oamphetamine (4-FA) and methoxetamine (MXE). In addition, these NPS were also reported most frequently to the Dutch Poisons Information Center by health care professionals (Hondebrink et al., 2015a).

Exposure to NPS results in many desired effects, such as euphoria, mental stimulation, intensification of sensory perception, increased sociability, increased energy, increased empathy, openness, less inhibitions, and sexual arousal (Miliano et al., 2016). However, most users are unaware of possible adverse effects, which depend on the specific NPS used. For many different NPS severe health effects have been reported, including confusion, psychosis, suicidal thoughts and extreme aggression. In addition, life-threatening neurological and cardiovascular effects have been reported, such as arrhythmias, reverse Takotsubo cardiomyopathy, myocardial infarction, brain hemorrhage, convulsion and coma (Scottish Government Social Research, 2014; Wijers et al., 2017; Hohmann et al., 2014; Madias, 2015; Al-Abri et al., 2014; Butterfield et al., 2015). As a result, 9% of all drug-related emergency department visits involved the use of NPS (EMCDDA, 2015a, 2015b). The actual number is likely higher, due to difficulties in detecting NPS in blood or urine samples of users. In addition, patients visiting the emergency department for a drug intoxication have high admission rates, reported up to 70% (Duineveld et al., 2012).

Commonly used illicit drugs are well known to increase extracellular brain levels of monoamines, including dopamine, norepinephrine and serotonin. Monoamine levels can be increased via vesicular release of monoamines, decreased breakdown of these neurotransmitters and via inhibition and/or reversal of monoamine reuptake transporters including the dopamine transporter (DAT), norepinephrine transporter (NET) and serotonin transporter (SERT) (Korpi et al., 2015). Such increased monoamines levels can be related to clinical outcomes. For example, increased dopaminergic activity is related to reinforcing and behavioral-stimulating effects of drugs (Kimmel et al., 2001; Volkow et al., 2009). Substances with a primary site of action at DAT are also known to have a high abuse liability and they can induce strong adverse effects (Howell and Kimmel, 2008; Koob and Volkow, 2010). On the other hand, increased adrenergic activity can induce a wide range of cardiovascular effects such as tachycardia, hypertension and hyperthermia (Greene et al., 2008). Finally, increased serotonergic activity can induce entactogenic effects, but can also result in adverse effects including the potentially life-threatening serotonin syndrome (Mugele et al., 2012).

Cocaine, amphetamine and MDMA are known inhibitors of monoamine transporters. In addition, amphetamine and MDMA can also induce reversal of membrane transporters, thereby further increasing extracellular brain levels of monoamines (Torres et al., 2003; Fleckenstein et al., 2007; Verrico et al., 2007; Rietjens et al., 2012). Since many NPS have molecular structures comparable to illicit drugs and also induce comparable intended effects, their mechanisms of action likely overlap. In support of this, inhibition and reversal of monoamine transporters has been reported for several NPS (Eshleman et al., 2013; Nagai et al., 2007; Rickli et al., 2015a, 2015b; Simmler et al., 2013, 2014). Since both the use and the number of available NPS (currently over 600, UNODC, 2016) are increasing and severe adverse health effects have been reported, there is an urgent need to rapidly assess the hazard and risk for human health.

Several assays can be used to determine the (neurotoxic) effects of NPS. Preferably, applied assays allow for rapid screening of a large number of substances. For example, effects on neuronal activity can be determined with considerable throughput using multi-well micro-electrode arrays. Recently, it was shown that common illicit drugs and NPS reduce neuronal activity at concentrations relevant for human exposure (Hondebrink et al., 2016). This integrated endpoint provides valuable information, but provides limited insight in the mechanisms of action. Targeted assays allow for investigation of specific mechanisms, including drug-induced effects on GABA receptors (Hondebrink et al., 2011a; Hondebrink et al., 2013; Hondebrink et al., 2015b), voltage-gated calcium channels (Hondebrink et al., 2011b; Hondebrink et al., 2012) or acetylcholine receptors (Hondebrink et al., 2012). Moreover, the function of monoamine reuptake transporters is often investigated as a mechanism of action for psychoactive drugs, including NPS. Assays measuring transporter function often rely on measurement of the uptake of radio-labelled transporter ligands by e.g. human embryonic kidney (HEK) cells transfected with the transporter of interest. To perform such assays, specific laboratory requirements are needed for handling radio-labelled material. In addition, this method only allows for examining effects at the end of a particular exposure, precluding real-time kinetic measurements during drug exposure.

In addition to radiometric assays, neurotransmitter transporter uptake activity can be measured using fluorescent substrates such as 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP$^+$), 4-(4-dimethylamino)-phenyl-1-methylpyridinium (APP$^+$), 1-methyl-4-phenylpyridium (MPP$^+$) and fluorescent false neurotransmitters (FFN) (Oz et al., 2010; Karpowicz et al., 2013; Schwartz et al., 2003; Fowler et al., 2006). Recently, a commercially available method was described using a fluorescent transporter substrate combined with a masking dye. Innovative aspects of this assay are that it does not require specific laboratory facilities or techniques, which makes it easy to use with a lower labor intensity. Also, high-throughput and real-time kinetic measurements can be performed using a plate reader (Jørgensen et al., 2008; Bernstein et al., 2012). The possibility to measure over time, for example, allows to investigate the reversibility of a drug-induced effect by adding potential antidotes, which is not possible using radiometric assays. Despite its benefits compared to radiometric assays, this method has rarely been used to measure effects of illicit drugs or NPS on the activity of neurotransmitter transporters. If this assay is applicable, it could aid in classifying NPS and quickly provide information on their mechanism of action.

The current research therefore investigates the applicability of this novel fluorescent assay to determine the potency of drugs, including NPS (Fig. 1), to inhibit monoamine reuptake transporters in comparison to radiometric assays.

2. Methods

2.1. Chemicals

Cocaine and DL-amphetamine hydrochloride salts (purity > 98.5%) were obtained from Spruyt Hillen (IJsselstein, the Netherlands). 3,4-Methylenedioxy-N-methylamphetamine (MDMA), 1-(4-fluorophenyl)-propan-2-amine (4-FA), 1-(4-methoxyphenyl)-N-methyl-propan-2-amine (PMMA), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2-(3-methoxyphenyl)-2-(ethylamino)cyclohexanone (MXE), 2-(4-bromo-2,5-dimethoxyphenyl)-N-((2-methoxyphenyl)methyl)ethanamine (25B-NBOMe), 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), (*RS*)-1-phenyl-2-(1-pyrrolidinyl)-1-pentanone (α-PVP) and 5-(2-aminopropyl)benzofuran (5-ABP) hydrochloride salts (purity > 98.5%) were obtained from Lipomed (Weil am Rhein, Germany). Poly-L-lysine hydrobromide (PLL) and fluoxetine were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). All other chemicals were purchased from Life Technologies (Bleiswijk, the Netherlands) unless otherwise stated. Hank's Balanced Salt Solution (1 ×) (HBSS) buffer solution in H_2O (cell culture grade) was prepared with addition of 20 mM HEPES. Drug stock solutions (2 or 100 mM) were prepared in HBSS (1 ×) and stored at 4 °C for a maximum of 4 days.

2.2. HEK 293 cell culture

Human embryonic kidney (HEK) 293 cells stably expressing human DAT, NET or SERT (kindly provided by Dr. Hoener from F. Hoffmann-La Roche Ltd., Basel, Switzerland) and non-transfected HEK-cells were
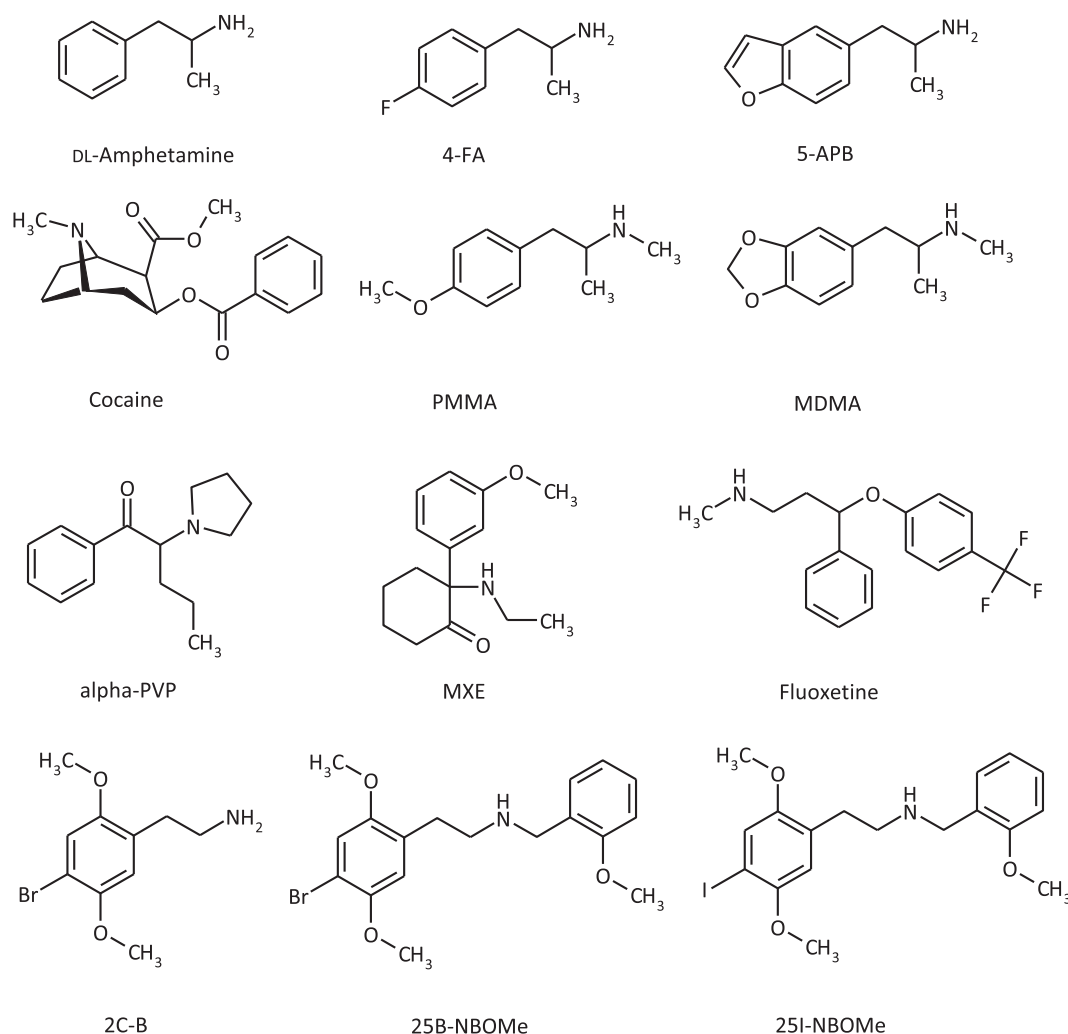
Fig. 1. Chemical structures of the tested substances including commonly used drugs (DL-amphetamine, cocaine and MDMA), NPS (4-FA, α-PVP, PMMA, 5-APB, 2C-B, 25B-NBOMe, 25I-NBOMe and MXE) and fluoxetine (see list of abbreviations or methods for full names).

cultured in T75 flasks (Thermo Fisher Scientific, Massachusetts, USA) at 37 °C and 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (41965-039) was supplemented with 10% dialyzed fetal bovine serum (FBS), 2 mM L-glutamine, 1% 5000 U/mL–5000 μg/mL penicillin/streptomycin, 1 mM sodium pyruvate, 1% minimum essential medium non-essential amino acids solution (MEM-NEAA) solution and 5 μL/mL geneticin selective antibiotic. Trypsin-EDTA (0.05%) was prepared by diluting trypsin-EDTA (0.5%) in phosphate-buffered saline (PBS). All other cell culture materials were obtained from Gibco (Life Technologies, Breda, The Netherlands). Medium was refreshed every 2–4 days and cells were passaged at > 80% confluence with the use of PBS and trypsin-EDTA (0.05%). Cells were used for up to 10 passages.
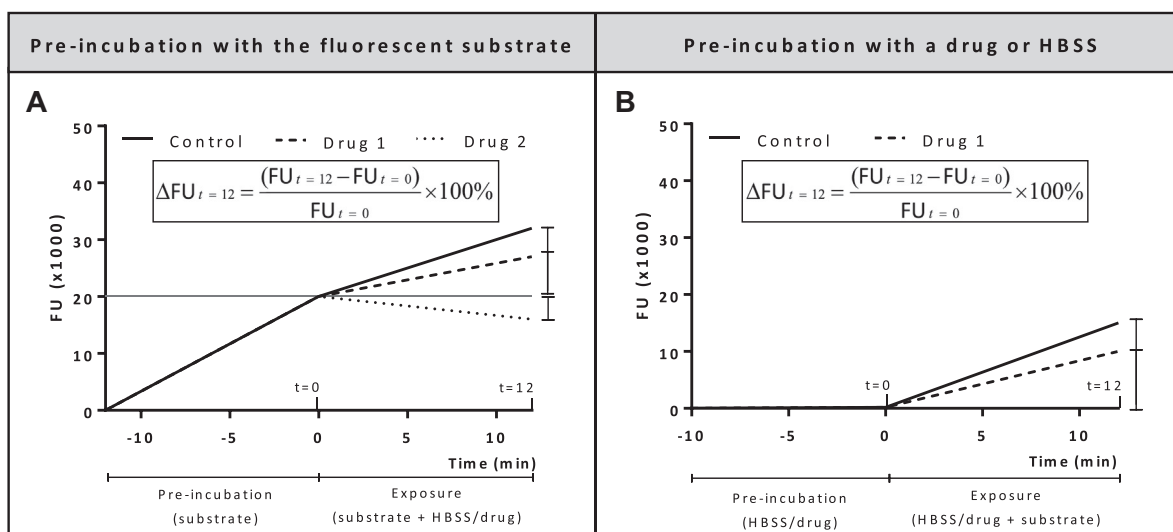
2.3. Inhibition of uptake by monoamine transporters

Uptake activity of hNET, hDAT and hSERT was measured using the Neurotransmitter Transporter Uptake Assay Kit from MDS Analytical Technologies (Sunnyvale, CA). The kit contained a mix consisting of a fluorescent substrate, which resembles the biogenic amine neurotransmitters, and a masking dye that extinguishes extracellular fluorescence. This product is patented by the manufacturer and the exact identity of the fluorescent substrate and masking dye therefore remains unknown. Uptake of the fluorescent substrate increases intracellular fluorescence, while extracellular fluorescence is blocked by the masking dye (Jørgensen et al., 2008). The fluorescent substrate solution was

prepared by dissolving the mix in HBSS according to the protocol provided by the supplier and stored at − 18 °C for a maximum of 4 days.

2.3.1. Drug-induced monoamine transporter uptake inhibition (pre-incubation with the fluorescent substrate)

On day 0, HEK 293 cells were seeded at a density of approximately 60.000 cells/well in clear-bottom, black-walled, 96-well plates (Greiner Bio-one, Solingen Germany) coated with PLL buffer (50 mg/L). Cells were allowed to proliferate overnight in a humidified 5% CO_2/95% air atmosphere at 37 °C. Experiments were performed the next day (day 1). Cells were pre-incubated with the fluorescent substrate for 12 min prior to a 30 min drug exposure ($t = − 12$ to $t = 0$). Culture medium was replaced by 100 μL/well fluorescent substrate solution, and uptake measurements were started. At $t = 0$, 100 μL/well HBSS without (control) or with drug was added to each well and uptake was measured continuously for 30 min. Background wells were pre-incubated with 100 μL/well HBSS without fluorescent substrate solution and exposed at $t = 0$ min to 100 μL/well HBSS without drugs. Non-transfected HEK 293 cells pre-incubated with 100 μL/well fluorescent substrate solution and exposed at $t = 0$ to 100 μL/well HBSS without drugs served as negative controls. Drugs (Fig. 1) were prepared daily in HBSS from 2 or 100 mM stock solutions. Cocaine, DL-amphetamine, MDMA, 4-FA, MXE, PMMA, 2C-B, 25B-NBOMe, 25I-NBOMe, α-PVP and 5-APB were measured at final concentrations of 0.01–1000 μM. For 25B-NBOMe and

Fig. 2. Fictional calculation of the change in fluorescence (ΔFU) (i.e. the uptake by monoamine transporters over time) per well. The relative monoamine uptake is determined in experiments where cells were pre-incubated with the fluorescent substrate (A) or with a drug or HBSS (B). The percentage uptake is first determined per well by calculating the change in fluorescence (ΔFU) at 12 min after drug/HBSS and substrate exposure (t = 12) compared to the fluorescence prior to the start of this exposure (t = 0) (i.e. the fluorescence following 12 or 10 min pre-incubation with the fluorescent substrate (A) or a drug/HBSS (B) respectively), as a percentage of the fluorescence prior to exposure (t = 0). Secondly, uptake in drug-exposed wells was expressed as a percentage of control wells (not shown in figure). The fictional effects of the addition of HBSS (Control; A), a drug inhibiting transporter function (Drug 1; A) and a drug reducing fluorescence lower than prior the fluorescence to exposure (for details see discussion)(Drug 2; A) to cells pre-incubated with the fluorescent substrate are shown. The fictional effects on monoamine uptake of cells pre-incubated with HBSS (Control) or drug (Drug 1) before the addition of the fluorescent substrate are also depicted (B).

25I-NBOMe the maximum concentration tested was 100 μM, as higher concentrations were cytotoxic. While continuous measurements (with temporal resolution determined by the speed of the plate reader) are possible, we measured fluorescence every 3 min, starting directly after addition of the fluorescent substrate solution (t = − 12). Fluorescence was measured with a microplate reader (Tecan Infinite M200 microplate; Tecan Trading Männedorf, Switzerland) at 37 °C at 430/515 nm excitation/emission wavelength in bottom-reading mode using optimal gain values for each cell type (number of cycles: 21, time interval: 3 min, number of flashes: 19, integration time: 20 μs, no lid). Cell attachment was visually examined following experiments.

2.3.2. Drug-induced monoamine transporter uptake inhibition (pre-incubation with drugs)

In many radiometric assays, cells are pre-incubated with drugs prior to incubation with the radio-labelled substrate. We therefore also tested this experimental condition using MDMA and cocaine as reference chemicals. On day 1, medium was removed and 100 μL/well HBSS without (control) or with MDMA or cocaine was added to each well for 10 min prior to addition of 100 μL fluorescent substrate solution/well. Following addition of the fluorescent substrate solution (t = 0), fluorescence was measured every 3 min for 30 min as described above.

2.4. Possible drug-induced reversal of monoamine transporters

Single-cell imaging was performed to investigate if the fluorescent substrate can be released via reverse transport. Changes in fluorescence of hSERT-transfected cells were measured at room temperature with the Neurotransmitter Transporter Uptake Assay Kit from MDS Analytical Technologies (Sunnyvale, CA). On day 0, cells were seeded on PLL-coated glass-bottom dishes (MatTek, Ashland, Massachusetts) at a density of 18.000 cells/dish. On day 1, medium was replaced with 300 μL buffer consisting of 50% fluorescent substrate solution and 50% HBSS comparable to the plate reader experiments. The dish was placed on the stage of an Axiovert 35 M inverted microscope (40 × oil-immersion objective, NA 1.0; Zeiss, Göttingen, Germany), equipped with a TILL Photonics Polychrome IV (Xenon Short Arc lamp, 150 W; TILL Photonics, GmBH, Gräfelfing, Germany). Fluorescence was measured every 3 min at 430/515 nm excitation/emission wavelength using an

Image SensiCam digital camera (TILL Photonics GmBH). Cells were pre-incubated with the fluorescent substrate solution for 21 min (t = − 21 to t = 0), after which the fluorescent substrate was removed and cells were washed with 1 mL HBSS (t = 0). Subsequently, HBSS was replaced by 500 μL HBSS without (control experiments) or with MDMA (1 mM). Fluorescence was continuously measured in single cells and in areas without cells for 30 min following exposure.

2.5. Estimated drug concentration in the brain

The estimated brain concentrations were calculated using human recreational serum/blood levels obtained from literature (voluntary intake, driving under the influence or non-fatal intoxications, except for 5-APB which was derived from human overdose cases). Next, a brain partitioning factor (BPF) was determined for each drug by dividing the brain concentration by the serum/blood concentration found in human post mortem or animal studies. These human (recreational) serum/blood levels were multiplied with the corresponding BPF to estimate human brain levels resulting from recreational drug use.

2.6. Data analysis

2.6.1. Calculating inhibition of uptake by monoamine transporters (in control experiments)

The fluorescence of each well was background corrected (time- and plate-matched). Linearity of the uptake curves (raw data, fluorescence units (FU)) was assessed for hDAT, hNET, hSERT and non-transfected HEK cells using linear regressions (GraphPad Prism, version 6.05). Linearity was assessed in cells pre-incubated only with the fluorescent substrate (t = − 12 to t = 0) as well as when HBSS without drugs was added after 12 min of pre-incubation with the fluorescent substrate (t = 0 to t = 30), comparable to the actual experimental conditions.

2.6.2. Calculating drug-induced monoamine transporter uptake inhibition (pre-incubation with the fluorescent substrate)

The fluorescence of each well was background corrected (time- and plate-matched). Uptake of the fluorescent substrate was first determined per well by calculating the change in fluorescence (ΔFU) at 12 min after drug exposure (t = 12) compared to the fluorescence prior

to exposure (i.e. the fluorescence following 12 min pre-incubation with the fluorescent substrate at $t = 0$), as a percentage of the fluorescence prior to exposure (Fig. 2A). Notably, as cells were pre-incubated with fluorescent substrate solution, fluorescence following drug exposure can be below the fluorescence prior to drug exposure and changes in fluorescence can therefore be negative (Fig. 2).

Secondly, the percentage uptake in control wells of all plates was averaged and wells that showed values $2 \times$ SD above or below average were considered as outliers and were excluded from further analysis (2%). Uptake in drug-exposed wells was expressed as a percentage of control wells. Outliers in exposed groups (effects $2 \times$ SD above or below average) were removed (2%) and data was expressed as the mean $\pm$ SEM of n wells obtained from at least 3 independent experiments (N plates) (cell were seeded from different passages or different thawings), with at least 3 wells (n) per plate. Concentration-response curves were made for each transporter and each exposure. IC_{50} values for multiple time points after exposure were based on full concentration-response curves (GraphPad Prism, version 6.05).

2.6.3. Calculating drug-induced monoamine transporter uptake inhibition (pre-incubation with drugs)

In a separate set of experiments, cells were pre-incubated with MDMA or cocaine for 10 min prior to addition of the fluorescent substrate ($t = -10$ to $t = 0$). The following 12 min ($t = 0$ to $t = 12$), cells were exposed to both the drug and the fluorescent substrate. The fluorescence of each well was background corrected (time- and plate-matched). Uptake of the fluorescent substrate was first determined per well by calculating the change in fluorescence (ΔFU) at 12 min after drug and substrate exposure ($t = 12$) compared to the fluorescence prior to the drug and substrate exposure (i.e. the fluorescence following 10 min pre-incubation with the drug ($t = 0$), as a percentage of the fluorescence just after addition of the fluorescent substrate ($t = 0$) (Fig. 2B). Secondly, these values were analyzed as mentioned above, starting from averaging uptake in control wells of all plates.

2.6.4. Calculating possible drug-induced reversal of monoamine transporters

Single cells were incubated with fluorescent substrate for 18 min after which the fluorescent substrate was removed ($t = -18$ to $t = 0$). Subsequently, MDMA or HBSS (control) was added for 30 min. The fluorescence over time was analyzed in single cells and in areas without cells (background fluorescence). ΔFU in single cells at 6, 12 and 30 min after drug exposure ($t = 6$, 12 and 30) was corrected for background fluorescence and normalized to the fluorescence at $t = 0$. A possible change in background fluorescence was also analyzed. Data is expressed as mean $\pm$ SEM from n cells, obtained from N dishes.

3. Results

3.1. Inhibition of uptake by monoamine transporters (in control experiments)

To determine the effects of drugs on fluorescent substrate uptake, cells were pre-incubated with fluorescent substrate solution for 12 min, during which FU increased in control wells for all three transporters (Fig. 3). At $t = 0$, 100 μL HBSS was added, which diluted the extracellular fluorescent substrate concentration and slowed down the increase in FU. As illustrated in Fig. 3, ΔFU at 6 min after addition of HBSS ($t = 6$) was increased by 18% $\pm$ 0.3 ($n = 204$ wells, $N = 27$ plates), 20% $\pm$ 0.2 ($n = 167$ wells, $N = 22$ plates) and 28% $\pm$ 0.3 ($n = 182$ wells, $N = 23$ plates), respectively for hDAT, hNET and hSERT. ΔFU at 12 and 30 min after the addition of HBSS ($t = 12$ and $t = 30$) was 29% $\pm$ 0.4 and 54% $\pm$ 0.6 (hDAT), 34% $\pm$ 0.4 and 62% $\pm$ 0.6 (hNET), and 55% $\pm$ 0.5 and 109% $\pm$ 1.1 (hSERT) respectively. Linearity was assessed for the uptake curves from $t = -12$ to $t = 0$ (i.e. during pre-incubation with the substrate) and from $t = 0$
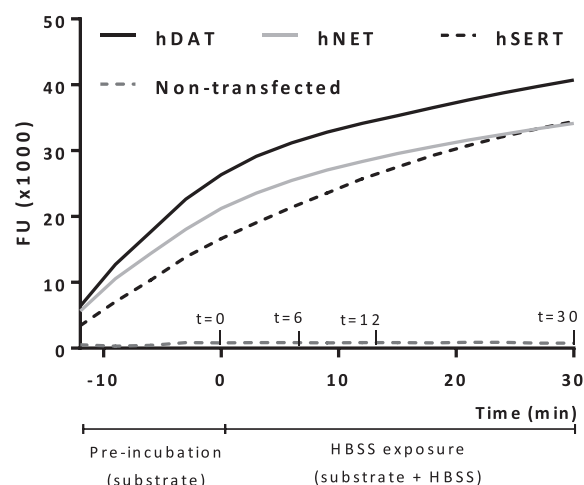

Fig. 3. Kinetic uptake curves of monoamine transporters. Uptake of fluorescent substrate, represented by the increase in FU over time, of hDAT, hNET and hSERT-expressing HEK cells ($n = 167$–204 wells, $N = 22$–27 plates) and non-transfected HEK cells ($n = 12$ wells, $N = 1$ plate) before ($t = -12$ to $t = 0$) and after the addition of 100 μL HBSS at $t = 0$ min ($t = 0$ to $t = 30$). The time points at which ΔFU was calculated are depicted by $t = 6$, $t = 12$ and $t = 30$.

to $t = 12$ (i.e. during exposure to HBSS). For all transporters, linearity was observed for both parts of the curve (for $t = -12$ to $t = 0$, R^2: 0.9918 (hDAT), 0.9930 (hNET) and 0.9982 (hSERT); for $t = 0$ to $t = 30$, R^2: 0.9740 (hDAT), 0.9698 (hNET) and 0.9843 (hSERT)). The linearity of the slope in the exposure window indicates that, while the fluorescent substrate concentration affects the rate of uptake, uptake remains linear allowing reliable assessment of drug-induced inhibition of reuptake transporters.

Non-transfected HEK cells did not show an increase in fluorescence (Fig. 3; $n = 12$ wells, $N = 1$ plate), indicating that the increase in fluorescence in cells transfected with transporters is due to transporter function and not to passive diffusion (See also Jørgensen et al. (2008) for additional uptake characteristics of the fluorescent substrate).

3.2. Drug-induced monoamine transporter uptake inhibition (pre-incubation with the fluorescent substrate)

Exposure to NPS and commonly used illicit drugs concentration-dependently inhibited uptake of monoamine transporters following 12 min of exposure (Fig. 4; Table 1). Cocaine potently inhibited all three transporters with IC_{50} values of 1.3–1.9 μM. α-PVP was over ten times more potent than cocaine in inhibiting uptake of hDAT and hNET (IC_{50} 0.1 μM), although α-PVP only weakly inhibited hSERT. DL-Amphetamine also potently inhibited hNET and to a lesser extent hDAT, but only weakly inhibited hSERT.

Stimulants that also are entactogenic showed a somewhat higher potency for inhibiting hSERT (IC_{50} 32–205 μM) compared to α-PVP and DL-amphetamine, but most potently inhibited hNET (IC_{50} 1.6–7.4 μM) and to a lesser extent hDAT (IC_{50} 7.7–83 μM).

The hallucinogenic compounds (25B-NBOMe, 25I-NBOMe and MXE) potently inhibited hSERT (IC_{50} 2.4–4.9 μM) and to a lesser extent hNET (IC_{50} 16–20 μM). These compounds inhibited hDAT only moderately (IC_{50} 33–137 μM). The hallucinogen 2C-B also preferentially inhibited hSERT, although with a ∼10-fold higher IC_{50} compared to 25B-NBOMe and 25I-NBOMe. As expected, the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine potently inhibited hSERT (IC_{50} 0.3 μM), whereas IC_{50} values for hNET and hDAT were respectively ∼25- and ∼450-fold higher.

Since the fluorescence-based assay allows for kinetic measurements, IC_{50} values for the different drugs on hDAT, hNET and hSERT were also calculated using different exposure durations. In addition to calculating IC_{50} values following 12 min of drug exposure ($t = 12$, Fig. 2), which is
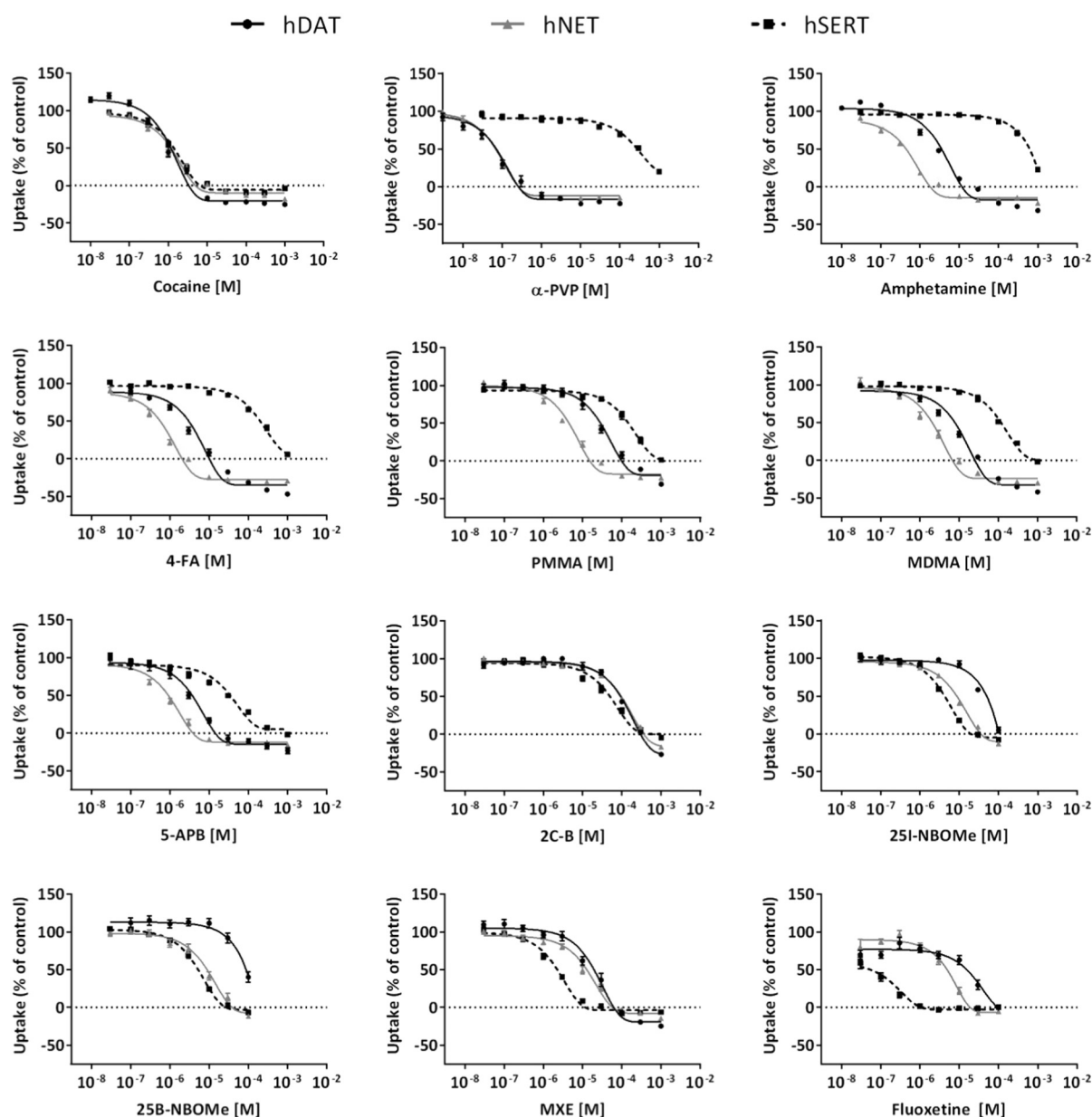
Fig. 4. Concentration-response curves of different drugs for the inhibition of uptake of fluorescent substrate via monoamine transporters (hDAT, hNET and hSERT) following 12 min of drug exposure. Prior to drug exposure, cells were pre-incubated with fluorescent substrate. Curves were fitted using nonlinear regression and data points are expressed as the mean ± SEM (n = 9–29 wells, N = 3–6 plates). The corresponding IC_{50} values can be found in Table 1.

comparable to the exposure duration generally used in radiometric assays, we also calculated IC_{50} values following 6 and 30 min exposure (t = 6 and t = 30). For most substances, IC_{50} values were comparable after 6, 12 and 30 min exposure (Supplemental Table 1).

3.3. Drug-induced monoamine transporter uptake inhibition (pre-incubation with drugs)

When transporter inhibition is investigated using radiometric assays, cells are often pre-incubated with the drug of interest prior to the addition of the radio-labelled substrate. Therefore, we also investigated this experimental condition using the fluorescent substrate for hSERT cells using cocaine and MDMA as reference chemicals. Cells transfected with hSERT were pre-incubated with cocaine or MDMA for 10 min prior to addition of the fluorescent substrate for 30 min to determine a possible difference in potency. Concentration-response curves of MDMA and cocaine when cells were pre-incubated with drugs before

fluorescent substrate addition were then compared to concentration-response curves where cells were pre-incubated with fluorescent substrate before drug exposure (Fig. 4). Data showed comparable concentration response curves (Fig. 5). The IC_{50} values at 6, 12 and 30 min after addition of the fluorescent substrate (t = 6, 12 and 30) were also comparable, although pre-incubation with drugs resulted in slightly higher IC_{50} values for MDMA (Supplemental Table 2).

3.4. Possible drug-induced reversal of monoamine transporters

To determine whether the fluorescence-based assay could detect reversal of monoamine transporters, single-cell imaging was used. This method is the preferred choice to study reversal as the sensitivity of the photodetectors of the imaging system exceeds that of the plate reader (at the expense of throughput). In addition, the single cell fluorescence imaging experiments allowed for measurement of the fluorescent intensity in the individual cells as well as in the surrounding medium

Table 1

Inhibition of monoamine transporter uptake by illicit drugs, NPS and fluoxetine. IC_{50} values (obtained following 12 min drug exposure) are presented with 95% confidence intervals [CI] (n = 9-29 wells, N = 3-6 plates). Grey blocks indicate the transporter(s) at which a compound is most potent.

Group	Drug	$IC_{50}(\mu M)$ [95% CI]		
		hDAT	hNET	hSERT
Stimulant / Entactogen / Hallucinogen	Cocaine	1.3 [1.1-1.5]	1.9 [1.5-2.4]	1.6 [1.4-1.8]
	α-PVP	0.1 [0.1-0.2]	0.1 [0.04-0.1]	>300
	Amphetamine	7.5 [6.3-8.9]	1.0 [0.8-1.2]	>300
	4-FA	21 [14-31]	1.8 [1.5-2.1]	205 [180-234]
	PMMA	83 [67-104]	7.4 [6.1-8.8]	180 [155-209]
	MDMA	43 [30-62]	4.4 [3.4-5.7]	121 [107-137]
	5-APB	7.7 [5.2-11]	1.6 [1.3-2.0]	32 [25-42]
	2C-B	240 [208-277]	166 [148-187]	54 [44-67]
	25I-NBOMe	75 [63-89]	19 [17-22]	4.3 [3.9-4.7]
	25B-NBOMe	137 [105-180]	16 [13-20]	4.9 [4.3-5.5]
	MXE	33 [23-48]	20 [15-27]	2.4 [2.0-2.8]
SSRI	Fluoxetine	136 [82-224]	7.9 [5.6-11]	0.3 [0.2-0.4]

(areas without cells). Since MDMA is known to reverse hSERT (Verrico et al., 2007; Rudnick and Wall, 1992; Mlinar and Corradetti, 2003), we investigated the effect of MDMA on single cells expressing hSERT.
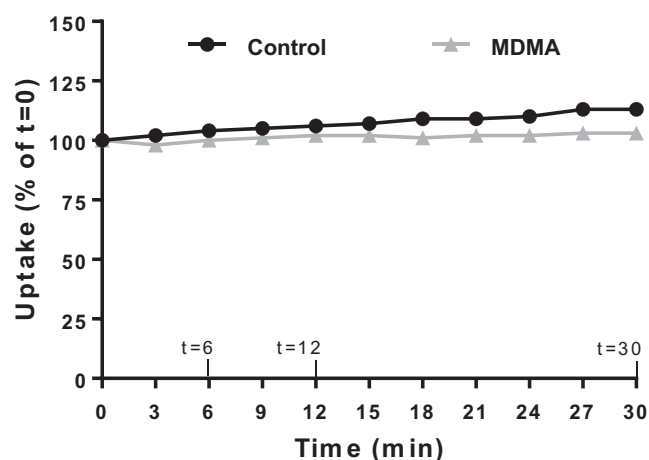
Following pre-incubation with fluorescent substrate, the substrate was removed and cells were exposed to HBSS or MDMA. In control experiments, a limited increase in fluorescence was observed at 6, 12 and 30 min after the replacement of the substrate with HBSS (t = 6, 12 and 30) of respectively 4% ± 2, 6% ± 2 and 13% ± 1, which was likely due to incomplete removal of the fluorescent substrate (Fig. 6). Following MDMA exposure (1 mM), a comparable, limited increase in fluorescence was observed at 6, 12 and 30 min of 0% ± 1, 2% ± 2 and 3% ± 2 respectively, which suggests that the fluorescent substrate is not subject to reverse transport. In addition, no increase in fluorescence was observed in the extracellular medium.

3.5. Estimated drug concentration in the brain

To determine if recreational drug use results in brain concentrations of NPS and drugs of abuse comparable to concentrations causing effects on monoamine transporters, human brain concentrations were estimated. Therefore, concentrations of drugs in serum and/or blood and brain partitioning factors (BPF) were gathered from literature (Table 2). Table 2 also shows which transporters are inhibited at the estimated human brain concentration. Almost all compounds inhibit at least one of the transporters at concentrations relevant for humans, except the NBOMe's,
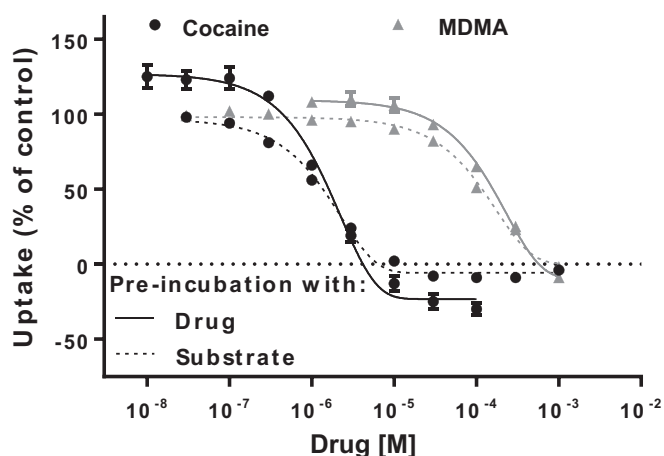
Fig. 5. Concentration-response curves of inhibition of hSERT by MDMA or cocaine using different experimental conditions. In one set of experiments, hSERT cells were pre-incubated for 12 min with fluorescent substrate (dotted line) prior to cocaine or MDMA exposure (12 min drug exposure; n = 14–16 wells, N = 4 plates). In the other set of experiments (solid line) cells were pre-incubated for 10 min with cocaine or MDMA prior to addition of fluorescent substrate (12 min fluorescent substrate exposure; n = 6 wells, N = 2 plates). Curves were fitted using nonlinear regression and are expressed as the mean ± SEM (due to small variation, the error bars are not always visible). Corresponding IC_{50} values can be found in Supplemental Table 2.

Fig. 6. The kinetic effect of MDMA exposure (1 mM) on hSERT-expressing cells pre-incubated with fluorescent substrate measured using single cell microscopy. Cells were exposed to MDMA (1 mM) or HBSS (control) for 30 min, following 18 min pre-incubation with fluorescent substrate, after which the fluorescent substrate was removed. Data was normalized to the fluorescence following pre-incubation with fluorescent substrate (t = 0) and expressed as the mean ± SEM (Control: n = 8 cells, N = 2 dishes, MDMA: n = 15 cells, N = 4 dishes).

Table 2

Estimated brain concentrations of commonly used illicit drugs, NPS and fluoxetine compared to their potency to inhibit mono-amine transporters. Estimated brain concentrations were calculated using human serum concentrations and brain partitioning factors (BPF) found in literature. All human serum concentrations were obtained from recreational use doses (voluntary intake, driving under the influence or accidental non-fatal intoxications), except for 5-APB. Serum concentration of α-PVP is based on blood concentrations (small caps). BPFs were based on serum and brain concentrations of rat (bold) or mouse (italic) data, or human post mortem blood (underlined) or serum (strikethrough) values compared to brain concentrations. Estimated brain concentrations for 5-APB and 25B-NBOMe were based on the observation that most BPFs are > 1. Grey blocks indicate that IC_{50} values obtained using the fluorescent assay are not in the estimated brain concentration range. Bold IC_{50} values indicate values within the estimated brain concentration range.

Group	Drug	Serum concentration (μM)	Brain partitioning factor (BPF)	Estimated brain concentration (μM)	IC_{50} (μM) hDAT/hNET/hSERT
Stimulant / Entactogen / Hallucinogen	Cocaine	0.2 - 1 [a,b,c,d,e]	4.5 - 5.5 [1,2]	0.9 - 5.5	**1.3/1.9/1.6**
	α-PVP	0.1 - 1 [i,j]	0.1 - 0.8 [6,7]	0.01 - 0.8	**0.1/0.1/>300**
	Amphetamine	0.1 - 8 [f,g,y]	8.5 - 12 [3,4,5]	0.9 - 96	7.5/**1.0**/>300
	4-FA	0.1 - 3 [h,x]	3 [15]	0.3 - 9	21/**1.8**/205
	PMMA	0.1 - 4 [l]	4 - 5.4 [9]	0.4 - 22	83/**7.4**/180
	MDMA	0.4 - 2 [k]	5 [8]	2 - 10	43/**4.4**/121
	5-APB	0.03 - 1 [m,n]	x	> 0.03 - 1	7.7/**1.6**/32
	2C-B	0.006 – 1.3 [o,p]	7 [10]	0.04 - 9	240/166/54
	25I-NBOMe	0.0006 - 0.007 [s]	7 [11]	0.004 - 0.05	75/19/4.3
	25B-NBOMe	0.0004 - 0.003 [q,r]	x	> 0.0004 - 0.003	137/16/4.9
	MXE	0.4 - 2 [t,u]	2.4 [12]	1 - 5	33/20/**2.4**
SSRI	Fluoxetine	0.2 - 0.4 [v,w]	20 - 23 [13,14]	4 - 9	136/**7.9**/**0.3**

References for serum concentrations: [a] Javaid et al., 1978; [b] Cone, 1995; [c] Jeffcoat et al., 1989; [d] Isenschmid et al., 1992; [e] Jenkins et al., 2002; [f] Lee et al., 2000; [g] Angrist et al., 1987; [h] Röhrich et al., 2012; [i] Eiden et al., 2013; [j] Wright and Harris, 2016; [k] de la Torre et al., 2000; [l] Vevelstad et al., 2012; [m] Elliot and Evans, 2014; [n] Adamowicz et al., 2014; [o] Adamowicz et al., 2016; [p] Ho et al., 2013; [q] Polkis et al., 2014a; [r] Laskowski et al., 2015; [s] Polkis et al., 2013; [t] Wood et al., 2012; [u] Shields et al., 2012; [v] Orsulak et al., 1988; [w] Rambourg Schepens, 1996; [x] Johansen and Hansen, 2012; [y] Holmgren et al., 2008. References for BPF calculation: [1] Brajkovic et al., 2016; [2] Bystrowska et al., 2012; [3] Rivière et al., 2000; [4] White et al., 2014; [5] Hendrickson et al., 2006; [6] Sykutera et al., 2015; [7] Hasegawa et al., 2014; [8] Mueller et al., 2009; [9] Pálenícek et al., 2011; [10] Rohanová et al., 2008; [11] Polkis et al., 2014b; [12] Horsley et al., 2016; [13] Karson et al., 1993; [14] Holladay et al., 1998; [15] Shiue et al., 1993.

4. Discussion

Our results obtained using an innovative, high-throughput, fluorescence-based assay demonstrate that 3 common illicit drugs, 8 NPS and the SSRI fluoxetine concentration-dependently inhibit human monoamine transporter function (hDAT, hNET and hSERT, Table 1, Fig. 4). Drugs were tested at non-cytotoxic concentrations (Rickli et al., 2015a, 2015b, 2015c; Simmler et al., 2013; Hondebrink et al., 2016). Stimulants such as amphetamine, 4-FA and PMMA in general selectively inhibit hNET and to a lesser extent hDAT. On the other hand, cocaine potently inhibits all three transporters, though α-PVP was over 10 times more potent on hNET and hDAT. In contrast, hallucinogenic drugs such as 2C-B, 25B-NBOMe, 25I-NBOMe and MXE were more potent on hSERT compared to hDAT and hNET. Thus, the fluorescence-based assay can effectively be used to investigate inhibition of human monoamine transporters by drugs. Neurotransmitter transporter inhibition profiles may be used to classify known and new drugs including NPS, thereby providing some information on possible adverse effects.

Importantly, inhibition of monoamine transporters was detected at concentrations relevant for human exposure. For almost all substances, the IC_{50} value for at least one of the transporters was in range of the estimated brain concentration, while four substances likely affect more than one transporter at estimated brain concentrations. Even the selective serotonin re-uptake inhibitor fluoxetine inhibits both hSERT (IC_{50} 0.3 μM) and hNET (IC_{50} 8 μM) at estimated therapeutic brain concentrations, in line with other literature (Karson et al., 1993; Renshaw et al., 1992; Komoroski et al., 1994; Strauss et al., 2002; Henry et al., 2005; Strauss and Dager, 2001). This suggests that transporter inhibition is a relevant mechanism of action during recreational use of these compounds. 2C-B, 25B-NBOMe and 25I-NBOMe had estimated brain concentrations below transporter inhibition, which may be explained by a potential underestimation of the estimated brain concentration due to the lack of information on brain partitioning. Also, NBOMe derivatives are potent 5-HT$_{2A}$ receptor agonists (nM range), which is currently considered as their main mechanism of action (Kyriakou et al., 2015).

Particular benefits of this fluorescence-based assay, compared to traditional radiometric assays, include the possibility to perform high-throughput and real-time kinetic measurements as well as its ease of use. Although we measured fluorescence only every 3 min, it is possible to measure continuously with temporal resolution limited only by the speed of the plate reader. In addition, due to kinetic measurements, each well can serve as its own internal control, i.e., baseline uptake can be established prior to drug exposure. The use of such internal controls reduces variation due to e.g. differences in transporter expression and/or cell numbers in different wells.

Moreover, kinetic measurements offer the possibility to determine the IC_{50} at different time points, thus allowing for more sophisticated experiments. For example, it is possible to pharmacologically modulate drug-induced inhibition in search for a possible antidote that may be applied in the treatment of intoxicated patients, which would not be possible if endpoint measurements like radiometric assays are used. HEK cells, lacking neuron-specific machinery, are an excellent model to investigate these direct effects on neurotransmitter function. Intoxicated patients might also benefit from this assay as that it can help to determine the most relevant mode of action of emerging NPS, providing some information on possible symptoms.

Since Ki values are hardly reported in literature, we compared the IC_{50} values we obtained with the fluorescence-based assay to those

Table 3

Inhibition of monoamine transporter uptake (IC$_{50}$, μM) by illicit drugs, NPS and fluoxetine compared to literature. All articles reported in this table used radio-labelled substrates. Potency for uptake inhibition was determined using transfected HEK293 cells (e, g, h, i, k, l, m, o, p, q), rat brain synaptosomes (a, b, f, g, j, n, italic), human platelets (c, SERT, underlined), C6 glial cells (c, DAT + NET, underlined with stripes), or JAr cells (d, underlined with dots). Almost all studies used cells in suspension, except for b, d, g, h, k and c, DAT + NET (bold).

Group	Drug	Reported IC$_{50}$(μM)					
		hDAT		**hNET**		**hSERT**	
		Our	*Literature*	*Our*	*Literature*	*Our*	*Literature*
Stimulant / Entactogen / Hallucinogen	Cocaine	**1.3**	0.5[e],*0.9[f]*, 0.4,[i] *0.2[j,n]*0.8[l]	**1.9**	0.3[e], *0.3[f]*, 0.2[i], *0.3[j,n]*, 0.5[l]	**1.6**	0.5[e], *2.1[f]*, 0.3[i], *0.3[j,n]*, 2.4[l]
	α-PVP	**0.1**	*0.01[n]*, 0.1[o]	**0.1**	*0.01[n]*, 0.02[o]	**>300**	*>10[n]*, >100[l]
	Amphetamine	**7.5**	*0.2[a], 0.1[j,n]*, **1.5[k]**, 1.3, 1.3[lo]	**1**	*0.2[a],0.1[j,n]*, **1.5[k]**, 0.1[l], 0.1[o]	**>300**	*3.8[a],3.4[j,n]*, **110[k]**, >10[l], 45[o]
	4-FA	**21**	*0.3[a]*, 0.8,[f]**10[k]**, 3.7[o]	**1.8**	*0.4[a]*, 0.4[f],**10[k]**,0.2[o]	**205**	*2.4[a]*, 6.8[f], **95[k]**, 19[o]
	PMMA	**83**	49[m]	**7.4**	1.2[m]	**180**	1.8[m]
	MDMA	**43**	<u>**0.5[c]**</u>, *1.4[f]*, 0.2,[i]10,[j] 17[l,m],31[o], 17[q]	**4.4**	<u>**2.1[c]**</u>, *0.7[f]*, 0.02[i], **12[k]**, 0.5[l,m],0.4[o], 0.36[q]	**121**	<u>1.4[c]</u>, *0.7[f]*, 0.1[i],**88[k]**, 1.4[l,m],2.0[o], 2.4[q]
	5-APB	**7.7**	6.1[q]	**1.6**	0.2[q]	**32**	0.3[q]
	2C-B	**240**	231[p]	**166**	44[p]	**54**	18[p]
	25I-NBOMe	**75**	65[p]	**19**	10[p]	**4.3**	6.8[p]
	25B-NBOMe	**137**	117[p]	**16**	6.7[p]	**4.9**	7.1[p]
	MXE	**33**	-	**20**	-	**2.4**	-
SSRI	Fluoxetine	**136**	*5[g], 8[g], 15[g], 210[h]*	**7.9**	**0.8[g], 0.5[g],** *0.2[g]*	**0.3**	**0.06[g], 0.02[g],** *0.01[g],0.01[b],* **0.05[d]**

References: [a]Marona-Lewicka et al., 1995; [b]Hyttel, 1982; [c]Cozzi et al., 1999; [d]Martel and Keating, 2003; [e]Meltzer et al., 2006; [f]Nagai et al., 2007; [g]Jørgensen et al., 2008; [h]Yoon et al., 2009; [i]Eshleman et al., 2013; [j]Baumann et al., 2013; [k]Rosenauer et al., 2013; [l]Simmler et al., 2013; [m]Simmler et al., 2014; [n]Marusich et al., 2014; [o]Rickli et al., 2015a; [p]Rickli et al., 2015b; [q]Rickli et al., 2015c.

reported measured with radio-labelled ligands. Notably, IC$_{50}$ values are mostly comparable between methods (Table 3). This is in line with previous studies that investigated the potency of antidepressants to inhibit transporter function and applied both the radiometric method and the fluorescent substrate. No difference in inhibition potencies was observed between both methods when experimental conditions like temperature or cell attachment were kept similar (Tsuruda et al., 2010; Jørgensen et al., 2008). For (illicit) drugs or NPS, only one article determined Ki values on hNET uptake using both a radiometric method (^{3}H-NE) and an analogous fluorescent substrate, ASP$^+$. Comparable Ki values were determined with both methods for amphetamine, cocaine and MDMA to inhibit hNET uptake (Haunsø and Buchanan, 2007). The IC$_{50}$ values, obtained using a fluorescent substrate, for the inhibition of hSERT for the phenethylamines amphetamine, 4-FA, MDMA and PMMA differ strongly compared to results obtained by others using radiometric methods (for references see Table 3). Differences in IC$_{50}$ values for transporter inhibition by drugs between our study and others could be due to the use of disparate experimental setups, such as cell type, cells in suspension versus attached cells, and measurements at room temperature versus at 37 °C.

Notably, in contrast to radio-labelled ligands, our single-cell imaging experiments, suggest that the fluorescent substrate is not subject to reverse transport (Fig. 6). The fluorescent substrate is therefore ideally suited to study inhibition of transporters as results are not confounded by reverse transport. Consequently, the fluorescence levels below zero at high drug concentrations (Fig. 4) were likely not caused by reverse transport and/or passive dye leakage. This effect is likely due to other factors, such as limited dye bleaching and/or limited sequestration of the dye in intracellular compartments with different ionic/pH conditions that somewhat attenuate the fluorescence of the dye. The fluorescence levels fluctuation around 100% at low drug concentrations are likely simply reflecting biological variation at a no-effect level.

Additional differences between our assay and those using radio-labelled substrates, relate to the experimental conditions. In our study, we aimed at mimicking in vivo conditions as closely as possible. Therefore, the cells were pre-incubated with fluorescent substrate prior to drug exposure providing intracellular levels of 'endogenous' substrate, which is more comparable to the in vivo situation. On the other hand, most studies using radio-labelled substrates pre-incubate the cells with drugs prior to adding the substrates. It has previously been suggested that pre-incubation of cells with 'slow binding' drugs results in lower IC$_{50}$ values (Tsuruda et al., 2010). Our data indicate that pre-incubating cells with drugs or pre-incubating cells with substrate has limited effects on the hSERT IC$_{50}$ values (Fig. 5 and Supplemental Table 2). Those particular experiments should be considered as proof of principle and were therefore limited to one transporter and two drugs. hSERT was chosen, since it showed the highest difference between IC$_{50}$ data measured using the fluorescence-based and radiometric-based assays, especially with exposure to phenethylamines. Therefore, a drug of the phenethylamines class (MDMA) and a non-phenethylamine drug (cocaine) were chosen.

Since uptake and binding for hSERT is known to be temperature dependent (Tsuruda et al., 2010; Elfving et al., 2001; Saldaña and Barker, 2004; Oz et al., 2010), our experiments were performed at a physiological temperature (37 °C), whereas most other studies performed experiments at room temperature. Notably, we observed a ~3 fold increase in MDMA potency on hSERT when temperature was lowered from 37 °C to room temperature (data not shown). Thus, the physiological temperature used in our study likely accounts for some of the observed differences in IC$_{50}$ values.

Furthermore, we used attached cells in our experiments, in contrast to most other studies that used cells in suspension (Table 3). To obtain cells in suspension, trypsin is often used. This process can cause changes in cell morphology and damage to membrane proteins, resulting in cellular dysfunction and stress responses (Huang et al., 2010), which may increase the sensitivity of cells to the effect of drugs. Only one study used attached cells at 37 °C to investigate illicit drugs and NPS using radio-labelled substrates. In line with our data, they also reported lower potencies for amphetamine, 4-FA and MDMA to inhibit hSERT (IC$_{50}$ ± 100 μM; Rosenauer et al., 2013). Simmler and Liechti (2016) also reported drugs to be less potent releasers when attached cells were used compared to cells in suspension.

Alternatively, the difference between our data and data found in literature may be explained by interaction of the fluorescent substrate with the binding site of phenethylamines at hSERT, since for non-phenethylamine drugs effects on hSERT were comparable to those reported with radiometric assays. However, since the manufacturer would not provide chemical details about the substrate, this could not be assessed.

Thus, many differences in the experimental approach could explain the difference between the IC$_{50}$ values of phenethylamines on hSERT inhibition measured using the fluorescent substrate and radioactively labelled monoamines. Even though our fluorescence measurements are closer to physiological conditions, which of both methods derives correct IC$_{50}$ values of phenethylamines on hSERT remains unclear, resulting in the risk to over- or underestimate the potency of illicit drugs and NPS to inhibit monoamine transporters.

In summary, our data demonstrate that the novel fluorescent-based method detects drug-induced inhibition of hDAT, hNET and hSERT. This high-throughput kinetic assay discriminates between a variety of commonly used illicit drugs and NPS that concentration-dependently inhibit the reuptake of monoamines with high reproducibility between experiments. For most drugs, IC$_{50}$ values are in the range of estimated brain concentrations, indicating that inhibition of monoamine transporters contributes to the psychological effects. Compared to radiometric assays, IC$_{50}$ values are comparable for hDAT, hNET and hSERT, with the exception of phenethylamines on hSERT, which show higher IC$_{50}$ values in the fluorescence-based assay. These differences however might be explained by experimental differences. The fluorescence-based assay has several advantages compared to the use of radio-labelled monoamines, including the possibility to measure effects kinetically (providing temporal information about transporter regulation and function), at physiological conditions (cell integrity, endogenous neurotransmitter concentration, temperature) and being less laborious without requiring specific laboratory facilities.

Conflict of interest

The authors do not have any conflict of interest.

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References

Adamowicz, P., Zuba, D., Byrska, B., 2014. Fatal intoxication with 3-methyl-*N*-methylcathinone (3-MMC) and 5-(2-aminopropyl)benzofuran (5-APB). Forensic Sci. Int. 24, 126–132. http://dx.doi.org/10.1016/j.forsciint.2014.10.016.

Adamowicz, P., Gieron, J., Lechowicz, W., Skulska, A., Tokarczyk, B., 2016. The prevalence of new psychoactive substances in biological material - a three-year review of casework in Poland. Drug Test. Anal. 8 (1), 63–70.

Al-Abri, S., Meier, K.H., Colby, J.M., Smollin, C.G., Benowitz, N.L., 2014. Cardiogenic shock after use of fluoroamphetamine confirmed with serum and urine levels. Clin. Toxicol. 52, 1292–1295.

Angrist, B., Corwin, J., Bartlik, B., Cooper, T., 1987. Early pharmacokinetics and clinical effects of oral D-amphetamine in normal subjects. Biol. Psychiatry 722, 1357–1368. http://dx.doi.org/10.1016/0006-3223(87)90070-9.

Baumann, M.H., Partilla, J.S., Lehner, K.R., Thorndike, E.B., Hoffman, A.F., Holy, M., Rothman, R.B., Goldberg, S.R., Lupica, C.R., Sitte, H.H., Brandt, S.D., Tella, S.R., Cozzi, N.V., Schindler, C.W., 2013. Powerful cocaine-like actions of 3,4-Methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive "bath salts" products. Neuropsychopharmacology 38, 552–562. http://dx.doi.org/10.1038/npp.2012.204.

Bernstein, A.I., Stout, K.A., Miller, G.W., 2012. A fluorescent-based assay for live cell, spatially resolved assessment of vesicular monoamine transporter 2-mediated neurotransmitter transport. J. Neurosci. Methods 15, 357–366. http://dx.doi.org/10.1016/j.jneumeth.2012.06.002.

Brajkovic, G., Babic, G., Stosic, J.J., Tomasevic, G., Rancie, D., Kilibarda, V., 2016. Fatal cocaine intoxication in a body packer. Vojnosanit. Pregl. 73, 198–201. http://dx.doi.org/10.2298/VSP141105022B.

Butterfield, M., Riguzzi, C., Frenkel, O., Nagdev, A., 2015. Stimulant-related Takotsubo cardiomyopathy. Am. J. Emerg. Med. 33 476.e1–476.e3.

Bystrowska, B., Adamczyk, P., Moniczewski, A., Zienwska, M., Fuxe, K., Filip, M., 2012. LC/MS evaluation of cocaine and its metabolites in different brain areas, peripheral organs and plasms in cocaine self-administered rats. Pharmacol. Rep. 64, 1337–1349. http://dx.doi.org/10.1016/S1734-1140(12)70931-3.

Cone, E.J., 1995. Pharmacokinetics and pharmacodynamics of cocaine. J. Anal. Toxicol. 19, 459–478. http://dx.doi.org/10.1093/jat/19.6.459.

Cozzi, N.V., Sievert, M.K., Shulgin, A.T., Jacobill, P., Rhuolo, A.E., 1999. Inhibition of plasma membrane monoamine transporters by β-ketoamphetamines. Eur. J. Pharmacol. 381, 63–69. http://dx.doi.org/10.1016/S0014-2999(99)00538-5.

Duineveld, C., Vroegop, M., Schouren, L., Hoedemaekers, A., Schouten, J., Moret-Hartman, M., Kramers, C., 2012. Acute intoxications: differences in management between six Dutch hospitals. Clin. Toxicol. 50, 120–128.

Eiden, C., Mathieu, O., Catala, P., 2013. Toxicity and death following recreational use of 2-pyrrolidino valerophenone. Clin. Toxicol. (Phila.) 51, 899–903. http://dx.doi.org/10.3109/15563650.2013.847187.

Elfving, B., Bjørnholm, B., Ebert, B., Knudsen, B.M., 2001. Binding characteristics of selective serotonin reuptake inhibitors with relation to emission tomography studies. Synapse 41, 203–211.

Elliot, S., Evans, J., 2014. A 3-year review of new psychoactive substances in casework. Forensic Sci. Int. 243, 55–60. http://dx.doi.org/10.1016/j.forsciint.2014.04.017.

EMCDDA, 2015a. New psychoactive substances in Europe: an update from the EU early warning system. http://www.emcdda.europa.eu/publications/2015/new-psychoactive-substances (accessed on 6.6.2016).

EMCDDA, 2015b. European drug report 2015: trends and developments. http://www.emcdda.europa.eu/publications/edr/trends-developments/2015 (accessed on 5.6.2016).

Eshleman, A.J., Wolfrum, K.M., Hatfield, M.G., Johnson, R.A., Murphy, K.V., Janowsky, A., 2013. Substituted methcathinones differ in transporter and receptor interactions. Biochem. Pharmacol. 85, 1803–1815. http://dx.doi.org/10.1016/j.bcp.2013.04.004.

Flash Eurobarometer 401, 2014. Young people and drugs. Report. Conducted by TNS Political & Social at the request of the European Commission, Directorate-General for Justice. http://www.drugsandalcohol.ie/22196/13/Eurobarometer%20401%20Young%20people%20and%20drugs%20-%20full%20report.pdf (accessed 6.5.2016).

Fleckenstein, A.E., Volz, T.J., Riddle, E.L., Gibb, J.W., Hanson, G.R., 2007. New insights into the mechanism of action of amphetamines. Annu. Rev. Pharmacol. Toxicol. 47, 681–698. http://dx.doi.org/10.1146/annurev.pharmtox.47.120505.105140.

Fowler, A., Seifert, N., Acker, V., Woehrle, T., Kilpert, C., de Saizieu, A., 2006. A non-radioactive high-throughput/high-content assay for measurement of the human serotonin reuptake transporter function in vitro. J. Biomol. Screen. 2006, 1027–1034.

Greene, S.L., Kerr, F., Braitberg, G., 2008. Review article: amphetamines and related drugs of abuse. Emerg. Med. Australas. 20, 391–402. http://dx.doi.org/10.1111/j.1742-6723.2008.01114.x.

Hasegawa, K., Suzuki, O., Wurita, A., Minakata, K., Yamagishi, I., Nozawa, H., Gonmori, K., Watanabe, K., 2014. Postmortem distribution of α-pyrrolidinovalerophenone and its metabolite in body fluids and solid tissues in a fatal poisoning case measured by LC-MS-MS with the standard addition method. Forensic Toxicol. 32, 225–234. http://dx.doi.org/10.1007/s11419-014-0227-8.

Haunsø, A., Buchanan, D., 2007. Pharmalogical characterization of a fluorescent uptake assay for the noradrenaline transporter. J. Biomol. Screen. 12, 378–384. http://dx.doi.org/10.1177/1087057107299524.

Hendrickson, H., Laurenzana, E., Owens, S.M., 2006. Quantitative determination of total methamphetamine and active metabolites in rat tissue by liquid chromatography with tandem mass spectrometric detection. AAPS J. 8, E709–E717. http://dx.doi.org/10.1208/aapsj080480.

Henry, M.E., Schmidt, M.E., Hennen, J., Villafuerte, R.A., Butman, M.E., Tran, P., Kerner,

L.T., Cohen, B., Renshaw, P.F., 2005. A comparison of brain and serum pharmacokinetics of r-fluoxetine and racemic fluoxetine: a 19-F MRS study. Neuropsychopharmacology 30, 1576–1583.

Ho, R.Y., Gerona, R., Olson, K.R., 2013. Clinical course of 4-bromo-2,5-dimethoxyphenylamine (2C-B) intoxication with laboratory confirmation. Clin. Toxicol. (Phila.) 51, 666–667.

Hohmann, N., Mikus, G., Czock, D., 2014. Effects and risks associated with novel psychoactive substances: mislabeling and sale as bath salts, spice, and research chemicals. Dtsch. Arztebl. Int. 111, 139–147.

Holladay, J.W., Michael, J.D., Yoo, S.D., 1998. Pharmacokinetics and antidepressant activity of fluoxetine in transgenic mice with elevated serum alpha-1-acid glycoprotein levels. J. Pharmacol. Exp. Ther. 26, 20–24.

Holmgren, A., Holmgren, P., Kugelberg, F.C., Jones, A.W., Ahlner, J., 2008. High re-arrest rates among drug-impaired drivers despite zero-tolerance legislation. Accid. Anal. Prev. 40, 534–540. http://dx.doi.org/10.1016/j.aap.2007.08.009.

Hondebrink, L., Meulenbelt, J., van Kleef, R.G.D.M., van den Berg, M., Westerink, R.H.S., 2011a. Modulation of human GABAA receptor function: a novel mode of action of drugs of abuse. Neurotoxicology 32, 823–827.

Hondebrink, L., Meulenbelt, J., Meijer, M., van den Berg, M., Westerink, R.H.S., 2011b. High concentrations of MDMA ('ecstasy') and its metabolite MDA inhibit calcium influx and depolarization-evoked vesicular dopamine release in PC12 cells. Neuropharmacology 61, 202–208.

Hondebrink, L., Meulenbelt, J., Rietjens, S.J., Meijer, M., Westerink, R.H.S., 2012. Methamphetamine, amphetamine, MDMA ('ecstasy'), MDA and mCPP modulate electrical and cholinergic input in PC12 cells. Neurotoxicology 33, 255–260.

Hondebrink, L., Tan, S., Hermans, E., van Kleef, R.G.D.M., Meulenbelt, J., Westerink, R.H.S., 2013. Addictive inhibition of human $\alpha_1\beta_2\gamma_2$ GABAA receptors by mixtures of commonly used drugs of abuse. Neurotoxicology 35, 23–29.

Hondebrink, L., Nugteren-van Lonkhuyzen, J.J., Van den Gouwe, D., Brunt, T.M., 2015a. Monitoring new psychoactive substances (NPS) in The Netherlands: data from the drug market and the Poisons Information Centre. Drug Alcohol Depend. 147, 109–115. http://dx.doi.org/10.1016/j.neuro.2016.04.020.

Hondebrink, L., Hermans, E.J.P., Schmeink, S., van Kleef, R.G.D.M., Meulenbelt, J., Westerink, R.H.S., 2015b. Structure-dependent inhibition of the human $\alpha_1\beta_2\gamma_2$ GABAA receptor by piperazine derivatives: a novel mode of action. Neurotoxicology 51, 1–9.

Hondebrink, L., Verboven, A.H.A., Drega, W.S., Schmeink, S., de Groot, M.W.G.D.M., van Kleef, R.G.D.M., Wijnolts, F.M., de Groot, A., Meulenbelt, J., Westerink, R.H., 2016. Neurotoxicity screening of (illicit) drugs using novel methods for analysis of microelectrode array (MEA) recordings. Neurotoxicology 55, 1–9. http://dx.doi.org/10.1016/j.neuro.2016.04.020.

Horsley, R.R., Lhotkova, E., Hajkova, K., Jurasek, B., Kuchar, M., Palenicek, T., 2016. Detailed pharmacological evaluation of methoxetamine (MXE), a novel psychoactive ketamine analogue — behavioural, pharmcokinetic and metabolic studies in the Wistar rat. Brain Res. Bull. 126, 102–110. http://dx.doi.org/10.1016/j.brainresbull.2016.05.002.

Howell, L.L., Kimmel, H.L., 2008. Monoamine transporters and psychostimulant addiction. Biochem. Pharmacol. 75, 196–217. http://dx.doi.org/10.1016/j.bcp.2007.08.003.

Huang, H.L., Hsing, H.W., Lai, T.C., Chen, Y.W., Lee, R.T., Chan, H.T., Lyu, P.C., Lin, S.T., Lin, C.W., Lai, C.H., Chang, H.T., Chou, H.C., Chan, H.L., 2010. Trypsin-induced proteome alteration during cell subculture in mammalian cells. J. Biomed. Sci. 17, 36. http://dx.doi.org/10.1186/1423-0127-17-36.

Hyttel, J., 1982. Citalopram – pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 6, 227–295.

Isenschmid, D.S., Fishman, M.W., Foltin, R.W., Caplan, Y.H., 1992. Concentration of cocaine and metabolites in plasma of humans following intravenous administration and smoking of cocaine. J. Anal. Toxicol. 16, 311–314. http://dx.doi.org/10.1093/jat/16.5.311.

Javaid, J.I., Fischman, M.W., Schuster, C.R., Dekirmenjian, H., Davis, J.M., 1978. Cocaine plasma concentration: relation to physiological and subjective effects in humans. Science 202, 227–228. http://dx.doi.org/10.1126/science.694530.

Jeffcoat, A.R., Perez-Reyes, M., Hill, J.M., Sadler, B.M., Cook, C.E., 1989. Cocaine disposition in humans after intravenous injection, nasal insufflation (snorting), or smoking. Drug Metab. Dispos. 17, 153–159.

Jenkins, A.J., Keenan, R.M., Henningfield, J.E., Cone, E., 2002. Correlation between pharmacological effects and plasma cocaine concentrations after smoked administration. J. Anal. Toxicol. 26, 82–392. http://dx.doi.org/10.1093/jat/26.7.382.

Johansen, S.S., Hansen, T.M., 2012. Isomers of fluoroamphetamines detected in forensic cases in Denmark. Int. J. Legal Med. 126, 541–547. http://dx.doi.org/10.1007/s00414-012-0671-0.

Jørgensen, S., Nielsen, E.O., Peters, D., Dyhring, T., 2008. Validation of a fluorescence-based high-throughput assay for the measurement of neurotransmitter transporter uptake activity. J. Neurosci. Methods 169, 168–176. http://dx.doi.org/10.1016/j.jneumeth.2007.12.004.

Karpowicz, R.J., Dunn, M., Sulzer, D., Sames, D., 2013. APP$^+$, a fluorescent analogue of the neurotoxin MPP$^+$, is a marker of catecholamine neurons in brain tissue, but not a fluorescent false neurotransmitter. ACS Chem. Neurosci. 15, 856–869. http://dx.doi.org/10.1021/cn400038u.

Karson, C.N., Newton, J.E.O., Livingston, R., Jolly, J.B., Cooper, J.B., Sprigg, J., Komoroski, R.A., 1993. Human brain fluoxetine concentrations. J. Neuropsychiatr. Clin. Neurosci. 5, 322–329. http://dx.doi.org/10.1176/jnp.5.3.322.

Kimmel, H.L., Carroll, F.I., Kuhar, M.J., 2001. Locomotor stimulant effects of novel phenyltropanes in the mouse. Drug Alcohol Depend. 65, 25–36. http://dx.doi.org/10.1016/S0376-8716(01)00144-2.

Komoroski, R.A., Newton, J.E.O., Cardwell, D., Sprigg, J., Pearce, J., Karson, C.N., 1994. In vivo ^{19}F spin relaxation and localized spectroscopy of fluoxetine in the brain. Magn. Reson. Med. 31, 204–211.

Koob, G.F., Volkow, N.D., 2010. Neurocircuitry of addiction. Neuropsychopharmacology 35, 217–238. http://dx.doi.org/10.1038/npp.2009.110.

Korpi, E.R., Hollander den, B., Farooq, U., Vashchinkina, E., Rajkumar, R., Nutt, R.D., Hyytiä, P., Dawe, G.S., 2015. Mechanisms of action and persistent neuroplasticity by drugs of abuse. Pharmacol. Rev. 46, 872–1004. http://dx.doi.org/10.1124/pr.115.010967.

Kyriakou, C., Marinelli, E., Frati, P., Santurro, A., Afxentiou, M., Zaami, S., Busardo, F.P., 2015. NBOMe: new potent hallucinogens-pharmacology, analytical methods, toxicities, fatalities: a review. Eur. Rev. Med. Pharmacol. Sci. 19, 3270–3281.

Laskowski, L., Elbakoush, F., Calvo, J., Exantus-Bernard, G., Fong, J., Polkis, J.L., Polkis, A., Nelson, L.S., 2015. Evolution of the NBOMes: 25c- and 25B- sold as 25I-NBOMe. J. Med. Toxicol. 11, 237–241. http://dx.doi.org/10.1007/s13181-014-0445-9.

Lee, M., Song, Y., Hwang, B., Chau, C., 2000. Determination of amphetamine and methamphetamine in serum via headspace derivatization solid-phase microextraction-gas chromatography-mass spectrometry. J. Chromatogr. A 896, 265–273. http://dx.doi.org/10.1016/s0021-9673(00)00596-3.

Madias, 2015. Takotsubo syndrome due to 4-fluoroamphetamine. Clin. Toxicol. (Phila.) 53, 136.

Marona-Lewicka, D., Rhee, G.S., Sprague, J.E., Nichols, D.E., 1995. Psychostimulant-like effects of p-fluoroamphetamine in the rat. Eur. J. Pharmacol. 287, 105–113. http://dx.doi.org/10.1016/0014-2999(95)00478-5.

Martel, F., Keating, E., 2003. Uptake of 1-methyl-4-phenylpyridinium (MPP+) by the JAR human placental choriocarcinoma cell line: comparison with 5-hydroxytryptamine. Placenta 24, 361–369. http://dx.doi.org/10.1053/plac.2002.0917.

Marusich, J.A., Antonazzo, K.R., Wiley, J.L., Blough, B.E., Partilla, J.S., Baumann, M.H., 2014. Pharmacology of novel synthetic stimulants structurally related to the "bath salts" constituent 3,4-methylenedioxypyrovalerone (MDPV). Neuropharmacology 87, 206–213. http://dx.doi.org/10.1016/j.neuropharm.2014.02.016.

Meltzer, P.C., Butler, D., Deschamps, J.R., Maddras, B.K., 2006. 1-(4-Methylphenyl)-2-pyrrolidin-1-pentan-1-one (Pyrovalerone) analogues: a promising class of monoamine uptake inhibitors. J. Med. Chem. 49, 1420–1432. http://dx.doi.org/10.1021/jm050797a.

Miliano, C., Serpelloni, G., Rimondo, C., Merue, M., Marti, M., De Luca, M.A., 2016. Neuropharmacology of New Psychoactive Substances (NPS): focus on the rewarding and reinforcing properties of cannabimimetics and amphetamine-like stimulants. Front. Neurosci. 10.

Mlinar, B., Corradetti, R., 2003. Endogenous 5-HT, released by MDMA through serotonin transporter- and secretory vesicle-dependent mechanisms, reduces hippocampal excitatory synaptic transmission by preferential activation of 5-HT1B receptors located on CA1 pyramidal neurons. Eur. J. Neurosci. 18, 1559–1571.

Mueller, M., Yuan, J., Felim, A., Neudorffer, A., Peters, F.T., Maurer, H.H., McCann, U.D., Largeron, M., Ricaurte, G.A., 2009. Further studies on the role of metabolites in (+/-)-3,4-methylenedioxymethamphetamine-induced serotonergic neurotoxicity. Drug Metab. Dispos. 3, 2079–2086. http://dx.doi.org/10.1124/dmd.109.028340.

Mugele, J., Nañagas, K.A., Tormoehlen, L.M., 2012. Serotonin syndrome associated with MDPV use: a case report. Ann. Emerg. Med. 60, 100–102. http://dx.doi.org/10.1016/j.annemergmed.2011.11.033.

Nagai, F., Nonaka, R., Satoh, H., Kamimura, K., 2007. The effects of non-medically used psychoactive drugs on monoamine neurotransmission in rat brain. Eur. J. Pharmacol. 559, 132–137. http://dx.doi.org/10.1016/j.ejphar.2006.11.075.

Orsulak, P.J., Kenney, J.T., Debus, J.R., Crowley, G., Wittman, P.D., 1988. Determination of the antidepressant fluoxetine and its metabolite norfluoxetine in serum by reversed-phase HPLC, with ultraviolet detection. Clin. Chem. 345, 1875–1878.

Oz, M., Libby, T., Kivell, B., Jaligam, V., Ramamoorthy, S., Shippenberg, T.S., 2010. Real-time, spatially resolved analysis of serotonin transporter activity and regulation using the fluorescent substrate, ASP$^+$. J. Neurochem. 114, 1019–1029. http://dx.doi.org/10.1111/j.1471-4159.2010.06828.x.

Pálenícek, T., Malíková, M., Rohanová, M., Novák, T., Horácek, J., Fujáková, M., Höschl, C., 2011. Behavioral, hyperthermic and pharmacokinetic profile of para-methoxymethamphetamine (PMMA) in rats. Pharmacol. Biochem. Behav. 98, 130–139. http://dx.doi.org/10.1016/j.pbb.2010.12.011.

Polkis, J.L., Charles, J., Wolf, C.E., Poklis, A., 2013. High-performance liquid chromatography tandem mass spectrometry method for the determination of 2CC-NBOMe and 25I-NBOMe in human serum. Biomed. Chromatogr. 27, 1794–1800. http://dx.doi.org/10.1002/bmc.2999.

Polkis, J.L., Nanco, C.R., Troendle, M.M., Wolf, C.E., Polkis, A., 2014a. Determination of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe) in serum and urine by high performance liquid chromatography with tandem mass spectrometry in a case of severe intoxication. Drug Test. Anal. 6, 764–769. http://dx.doi.org/10.1002/dta.1522.

Polkis, J.L., Devers, K.G., Arbefeville, E.F., Pearson, J.M., Houston, E., Polkis, A., 2014b. Postmortem detection of 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine] in fluids and tissues determined by high performance liquid chromatography with tandem mass spectrometry from a traumatic death. Forensic Sci. Int. 234, e14–e20. http://dx.doi.org/10.1016/j.forsciint.2013.10.015.

Rambourg Schepens, M.O., 1996. Fluoxetine (PIM 651). International programme on chemical safety poisons information monograph 651. http://www.inchem.org/documents/pims/pharm/pim651.htm (accessed 13.7.2016).

Renshaw, P.F., Guimaraes, A.R., Fava, M., Rosenbaum, J.F., Pearlman, J.D., Flood, J.G., Puopolo, P.R., Clancy, K., Gonzalez, R.G., 1992. Accumulation of fluoxetine and norfluoxetine in human brain during therapeutic administration. Am. J. Psychiatry 149, 1592–1594.

Rickli, A., Hoener, M.C., Liechti, M.E., 2015a. Monoamine transporter and receptor interaction profiles of novel psychoactive substances: para-halogenated amphetamines and pyrovalerone cathinones. Eur. Neuropsychopharmacol. 25, 365–376. http://dx.doi.org/10.1016/j.euroneuro.2014.12.012.

Rickli, A., Liethi, D., Reinisc, J., Buchy, D., Hoener, M.C., Liechti, M.E., 2015b. Receptor interaction profiles of novel *N*-2-methoxybenzyl (NBOMe) derivatives aof 2,5-dimethoxy-substituted phenethylamines (2C drugs). Neuropharmacology 99, 546–553. http://dx.doi.org/10.1016/j.neuropharm.2015.08.034.

Rickli, A., Kopf, S., Hoener, M.C., Liechti, M.E., 2015c. Pharmacological profile of novel psychoactive benzofurans. Br. J. Pharmacol. 172, 3412–3425. http://dx.doi.org/10.1111/bph.13128.

Rietjens, S.J., Hondebrink, L., Westerink, R.H.S., Meulenbelt, J., 2012. Pharmacokinetics and pharmacodynamics of 3,4-methylenedioxymethamphetamine (MDMA): interindividual differences due to polymorphisms and drug–drug interactions. Crit. Rev. Toxicol. 42, 854–876. http://dx.doi.org/10.3109/10408444.2012.725029.

Rivière, G.J., Gentry, W.B., Owens, S.M., 2000. Disposition of methamphetamine and its metabolite amphetamine in brain and other tissues in rats after intravenous administration. J. Pharmacol. Exp. Ther. 292, 1042–1047. http://dx.doi.org/10.1371/journal.pone.0052060.

Rohanová, M., Pálenícek, T., Balíková, M., 2008. Disposition of 4-bromo-2,5-dimethoxyphenethylamine (2C-B) and its metabolite 4-bromo-2-hydroxy-5-methoxyphenethylamine in rats after subcutaneous administration. Toxicol. Lett. 178, 29–36. http://dx.doi.org/10.1016/j.toxlet.2008.01.017.

Röhrich, J., Becker, J., Kaufmann, T., Zorntlein, S., Urban, R., 2012. Detection of the synthetic drug 4-fluoroamphetamine (4-FA) in serum and urine. Forensic Sci. Int. 215, 3–7. http://dx.doi.org/10.1016/j.forsciint.2011.04.004.

Rosenauer, R., Luf, A., Holy, M., Freissmuth, M., Schmid, R., Sitte, H.H., 2013. A combined approach using transporter-flux assays and mass spectrometry to examine psychostimulant street drugs of unknown content. ACS Chem. Neurosci. 4, 182–190. http://dx.doi.org/10.1021/cn3001763.

Rudnick, G., Wall, S.C., 1992. The molecular mechanism of "ecstasy" [3,4-methylenedioxymethamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. Proc. Natl. Acad. Sci. U. S. A. 89, 1812–1817.

Saldaña, S.N., Barker, E.L., 2004. Temperature and 3,4-methylenedioxymethamphetamine alter human serotonin transporter-mediated dopamine uptake. Neurosci. Lett. 354, 209–212.

Schwartz, J.W., Blakely, R.D., DeFelice, L.J., 2003. Binding and transport in norepinephrine transporters: real-time, spatially resolved analysis in single cells using a fluorescent substrate. J. Biol. Chem. 278, 9768–9777. http://dx.doi.org/10.1074/jbc.M209824200.

Scottish Government Social Research, 2014. New psychoactive substances – evidence review. Scottish Government Publications Website: http://www.gov.scot/Resource/0045/00457682.pdf (Accessed on 13-03-2017).

Shields, J.E., Dargan, P.I., Wood, D.M., Puchnarewicz, M., Davies, S., Waring, W.S., 2012. Methoxetamine associated reversible cerebellar toxicity: three cases with analytical confirmation. Clin. Toxicol. (Phila.) 50, 438–440. http://dx.doi.org/10.3109/15563650.2012.683437.

Shiue, C.Y., Shiue, G.G., Rysavy, J.A., Pleus, R.C., Huang, H., Bai, L.Q., Cornish, K.G., Sunderland, J.J., Frick, M.P., 1993. Fluorine-18 and carbon-11 labeled amphetamine analogs-synthesis, distribution, binding characteristics in mice and rats and a PET study in monkey. Nucl. Med. Biol. 20 (8), 973–981.

Simmler, L.D., Liechti, M.E., 2016. Interactions of cathinone NPS with human transporters and receptors in transfected cells. Curr. Top. Behav. Neurosci. 2016, 1–24. http://dx.doi.org/10.1007/7854_2016_20.

Simmler, L.D., Buser, T., Donzelli, M., Schramm, Y., Dieu, L.H., Huwyler, J., Chaboz, S., Hoener, M.C., Liechti, M.E., 2013. Pharmacological characterization of designer

cathinones in vitro. Br. J. Pharmacol. 168, 458–470. http://dx.doi.org/10.1111/j.1476-5381.2012.02145.x.

Simmler, L.D., Rickli, A., Schramm, Y., Hoener, M.C., Liechti, M.E., 2014. Pharmacological profiles of aminoindanes, piperazines, and pipradrol derivatives. Biochem. Pharmacol. 88, 237–244. http://dx.doi.org/10.1016/j.bcp.2014.01.024.

Strauss, W.L., Dager, S.R., 2001. Magnetization transfer of fluoxetine in the human brain using fluorine magnetic resonance spectrometry. Biol. Psychiatry 49, 798–802.

Strauss, W.L., Unis, A.S., Cowan, C., Dawson, G., Dager, S.R., 2002. Fluorine magnetic resonance spectroscopy measurement of brain fluvoxamine and fluoxetine in pediatric patients treated for pervasive developmental disorders. Am. J. Psychiatry 159, 755–7620.

Sykutera, M., Cychowska, M., Bloch-Buguslawska, E., 2015. A fatal case of pentedrone and α-pyrrolidinovalerophenone poisoning. J. Anal. Toxicol. 39, 324–329. http://dx.doi.org/10.1093/jat/bkv011.

de la Torre, R., Farré, M., Ortuño, J., Mas, M., Brenneisen, R., Roset, P.N., Segura, J., Cami, J., 2000. Non-linear pharmacokinetics of MDMA ('ecstasy') in humans. Br. J. Clin. Pharmacol. 49, 104–109. http://dx.doi.org/10.1046/j.1365-2125.2000.00121.x.

Torres, G.E., Gainetdinov, R.R., Caron, M.G., 2003. Plasma membrane monoamine transporters: structure, regulation and function. Nat. Rev. Neurosci. 4, 13–25. http://dx.doi.org/10.1038/nrn1008.

Tsuruda, P.R., Yung, J., Martin, W.J., Chang, R., Mai, N., Smith, J.A.M., 2010. Influence of ligand binding kinetics on functional inhibition of human recombinant serotonin and norepinephrine transporters. J. Pharmacol. Toxicol. Methods 61, 192–204. http://dx.doi.org/10.1016/j.vascn.2009.12.003.

UNODC, 2016. World drug report. In: United Nations Office on Drugs and Crime. Nations publication, United. https://www.unodc.org/wdr2016/en/index.html?lf=1&lng=en (accessed on 5.6.2016).

Verrico, C.D., Miller, G.M., Madras, B.K., 2007. MDMA (Ecstasy) and human dopamine, norepinephrine, and serotonin transporters: implications for MDMA-induced neurotoxicity and treatment. Psychopharmacology 189, 489–503. http://dx.doi.org/10.107/s00213-005-0174-5.

Vevelstad, M., Oiestad, E.L., Middelkoop, G., Hasvold, I., Lilleng, P., Delaveris, G.J.M., Eggen, T., Mørland, J., Arnestad, M., 2012. The PMMA epidemic in Norway: comparison of fatal and non-fatal intoxications. Forensic Sci. Int. 219, 151–157. http://dx.doi.org/10.1016/j.forsciint.2011.12.014.

Volkow, N.D., Fowler, J.S., Wang, G.J., Baler, R., Telang, F., 2009. Imaging dopamine's role in drug abuse and addiction. Neuropharmacology 56, 3–8. http://dx.doi.org/10.1016/j.neuropharm.2008.05.022.

White, S.J., Hendrickson, H.P., Atchley, W.T., Laurenzana, E.M., Gentry, W.B., Williams, D.K., Owens, S.M., 2014. Treatment with a monoclonal antibody against methamphetamine and amphetamine reduces maternal and fetal rat brain concentrations in late pregnancy. Drug Metab. Dispos. 42, 1285–1291. http://dx.doi.org/10.1124/dmd.114.056879.

Wijers, C.H.W., van Litsburg, R.T.H., Hondebrink, L., Niesink, R.J.M., Croes, E.A., 2017. Acute toxic effects related to 4-fluoroamphetamine. Lancet 389, 600.

Wood, D.M., Davies, S., Puchnarewicz, M., Johnston, A., Dargan, P.I., 2012. Acute toxicity associated with the recreational use of the ketamine derivative methoxetamine. Eur. J. Clin. Pharmacol. 68, 853–856. http://dx.doi.org/10.1007/s00228-011-1199-9.

Wright, T.H., Harris, C., 2016. Twenty-one cases involving alpha-pyrrolidinovalerophenone (α-PVP). J. Anal. Toxicol. 40, 396–402. http://dx.doi.org/10.1093/jat/bkw029.

Yoon, Y.S., Cho, T., Yoon, S.H., Min, C.K., Lee, C., 2009. *N*-methyl amine-substituted fluoxetine derivatives: new dopamine transporter inhibitors. Arch. Pharm. Res. 32, 1663–1671. http://dx.doi.org/10.1007/s12272-009-2201-2.